Tumorigenicity Risk of iPSCs in vivo: Nip it in the Bud

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Abstract

In 2006, Takahashi and Yamanaka first created induced pluripotent stem cells from mouse fibroblasts via the retroviral introduction of genes encoding the transcription factors Oct3/4, Sox2, Klf4, and c-Myc. Since then, the future clinical application of somatic cell reprogramming technology has become an attractive research topic in the field of regenerative medicine. Of note, considerable interest has been placed in circumventing ethical issues linked to embryonic stem cell research. However, tumorigenicity, immunogenicity, and heterogeneity may hamper attempts to deploy this technology therapeutically. This review highlights the progress aimed at reducing induced pluripotent stem cells tumorigenicity risk and how to assess the safety of induced pluripotent stem cells cell therapy product.

Keywords: induced pluripotent stem cells (iPSCs), tumorigenicity, regenerative medicine, reprogramming transcription factors, chemical induced reprogramming, drug-inducible suicide system
Introduction

Induced pluripotent stem cells (iPSCs), characterized by self-renewal and multiple differentiation potential, have been explored and applied in regenerative therapy, disease modeling, drug toxicity evaluation, and developmental biology. To date, transcription factor (TF) - mediated and chemical inductive reprogramming have been the strategies of choice to obtain iPSCs. Specifically, in TF-mediated reprogramming, the Yamanaka factors, Oct-4, Sox2, c-Myc, and Klf4 (OSMK), or alternative TFs (Table 1), are introduced into somatic cells with viral or non-viral vectors. In the chemical inductive reprogramming strategy, somatic cells are induced to become pluripotent stem cells through small molecules, cytokines, or growth factors. Romanazzo et al summarized the merits and demerits of these reprogramming strategies and pointed out that the TFs used in the transgenic strategy lead to random epigenetic events, the activation of various pluripotent genes (i.e., Oct-4 and c-Myc), and genomic instability (e.g., chromosomal aberrations, copy number variations, and single nucleotide variants). Such events were identified predominantly in iPSCs compared with ESCs, suggesting a potential link between iPSCs and tumorigenicity. Compared with the transgenic strategy, chemical induction seems to decrease the possibility of tumorigenesis. However, the lower reprogramming efficiency and the induction system's complexity are still obstacles to the chemical induction's clinical application.

Additionally, the heterogeneity of the cellular product derived from iPSCs for clinical therapy could lead to potential tumorigenicity risks in vivo. Theoretically, regardless of the strategy used to generate iPSCs, it may be impossible to avoid their heterogeneity due to the heterogeneity of the original somatic cells. Specifically, the established iPSC lines may still contain somatic cells and partially reprogrammed cells similar to iPSCs. Thus, the final cell preparation obtained may be a mixture of target cells for therapeutic use, the residual undifferentiated iPSCs or partially differentiated iPSCs with teratomas forming potential. The complicated in vivo environment makes cell integration and reproduction uncontrolled. Hence, decreasing the heterogeneity and increasing the controllability of iPSCs and cell preparation through isolation, purification, amplification, and modification of stem cells are necessary to decrease the tumorigenicity.
potential. Significant progress has been made over the last two decades in this research field, although many problems remain.

Finally, we focused on the current discussion on the safety assessment of cell therapy products (CTP) of iPSC, including genome integrity, heterogeneity and in vivo tumorigenicity. Although there are no mandatory provisions issued yet, the evaluation of iPSC genome integrity is recommended as one of the most important items because it presents a close association with the tumorigenicity of the iPSC products. To date, many alternative methods for checking the genetic mutations have become available. However, the cost of procedure, the complexity of result interpretation and the workload of data analysis have to be considered when the practical methods are picked up.

1 Optimizing the cocktail of reprogramming factors

As shown in Table 1, the past decade has seen the establishment of several combinations of TFs that can efficiently reprogram somatic cells based on the Yamanaka factors. Of these, c-Myc is the most controversial TF. It is well known that c-Myc is a proto-oncogene, encoding the family of beta helix-loop-helix/leucine zinc finger TFs, and its deregulated expression occurs in a wide range of human cancers, which lead to the discussion about the connection between c-Myc and iPSCs tumorigenicity. Hence, some researchers carried out iPSCs without c-Myc-based cell therapy to explore whether the absence of exogenous c-Myc can reduce iPSCs tumorigenic capacity without influencing the pluripotency. For example, Li et al. reported that 3-gene iPSCs (without c-Myc) differentiate into functional hepatocytes after receiving proper differentiation stimuli. iPSCs and iPSC-derived hepatocytes could decrease the thioacetamide (TAA)-induced hepatic necrosis of mice and restore liver function in mice with lethal acute hepatic failure (AHF) after undergoing intravenous or intrasplenic transplantation. This study highlights the potential of iPSCs, without c-Myc, in diminishing the incidence of tumorigenesis of cell transplantation.

Alternatively, it is reported that c-Myc paralogs, such as L-Myc and N-Myc can be used to reduce teratoma in the iPSCs. Nakagawa et al. chose L-Myc and c-Myc mutants (W136E c-Myc mutant and dN2 c-Myc mutant) to balance the efficiency and safety in reprogramming. Notably,
compared to the mice derived from Myc-minus iPSCs, those from L-Myc iPSCs did not present tumorigenicity. Importantly, although the mice from L-Myc-iPSCs exhibited slightly higher mortality, these alternatives from the Myc family are worth considering when obtaining iPSC cells with non/low teratogenicity from human somatic cells\(^\text{13}\).

In addition, some chemicals can replace specific TFs to help improve reprogramming efficiency\(^\text{16-19}\), but the systematic studies on the tumorigenicity of iPSCs derived from different cocktails are not sufficient. Pushp et al. argued that cocktails containing TFs and small molecules are better in reprogramming efficiency than chemical cocktails alone, exhibiting less tumorigenicity than TFs only\(^\text{20}\). The optimized formulas are listed in in Table 1. For instance, Maherali et al. demonstrated that ALK4/5/7 inhibitor SB-431542 replacing exogenous c-Myc improved the reprogramming efficiency of MEF\(^\text{21}\). Huangfu and colleagues discovered that Valproic acid (VPA), a histone deacetylase inhibitor, and DNA methyltransferase inhibitors facilitate MEFs and primary human fibroblast reprogramming processes\(^\text{22,23}\). Also, either RepSox or CHIR99021 substitute Sox2 and c-Myc\(^\text{16,17}\), the combination of Oct4, VPA, CHIR99021 and RepSox could induce reprogramming of MEF to form AP positive clones\(^\text{18}\). Unfortunately, there was no article showing the result about tumorigenicity of iPSCs derived from different cocktails.

### Table 1. Representative cocktail of TFs or TF-chemicals applied in generation of iPSCs.

| TFs                  | Chemicals | Efficiency          | Original Cell                  | Ref |
|----------------------|-----------|---------------------|--------------------------------|-----|
| OSKM                 | /         | 0.02%               | fibroblasts                     | 24  |
| OSNL                 | /         | 0.22%               | IMR90 fetal fibroblasts         | 2   |
| OSK & n-Myc/L-Myc    | similar to OSKM | ~11.8% ± 2.2% | MEF                            | 6   |
| OKM                  | /         | 0.001%~0.002%       | Mouse neural progenitor cells   | 25  |
| OSE                  | /         | ~50% of OSKM        | MEF                            | 26  |
| Oct3/4, Sox2, Klf4, c-Myc | VPA       | ~11.8% ± 2.2%       | MEF                            | 6   |
| OSK                  | /         | <0.001%             | Human dermal fibroblasts        | 12  |
| OSNL                 | /         | 0.01%               | Human newborn foreskin fibroblasts | 2   |
| OSK                  | VPA       | 1.1%                | Human neonatal foreskin fibroblasts | 23  |
| OS                   | VPA       | 0.004%              | Human neonatal dermal          | 23  |
2 Strategy of chemical-induced reprogramming

Studies have shown that cell fate reprogramming is comparably successful through the use of chemicals instead of the conventional gene transformation. As listed in Table 2, Deng and his team reported a series of significant achievements. Specifically, they obtained pluripotent stem cells (PSCs), named CiPSCs (chemically induced PSCs), through chemical inductive reprogramming, but also obtained transdifferentiated cells directly from original somatic cells, known as direct reprogramming or lineage reprogramming. In addition, recent reports demonstrated that biochemical signals (e.g., cytokines, growth factors, ECM proteins, and small molecules) directly generated the target terminally differentiated cells from original somatic cells without making iPSCs in front. Li et al reported chemically induced the extra-embryonic endoderm (XEN)-like state can be induced to functional neurons and hepatocytes bypassing the pluripotent state. Such findings might lead to a decreased tumorigenesis risk of iPSCs. However, a related systematic study on the underlying mechanism and tumorigenesis risk has not been reported to date. Moreover, unlike TFs, chemicals are mostly synthetic with clear targets for regulating biologic activities, primarily through receptors and enzymes. More studies are needed to develop chemicals for reprogramming cell fate as reliably and rationally as TFs but without safety concerns. Chen et al. summarized the small molecule combinations that have been demonstrated with sufficient efficiency in somatic cell reprogramming and lineage reprogramming. It is worth to note that there are a few chemicals that promote reprogramming effectively through epigenetic modifications, such as histone deacetylase inhibitor TSA, SAHA and VPA.
(Valproic acid), DNMT (DNA methyltransferase) inhibitor 5-AZA and RG108, histone methyltransferase G9a (Bix-01294) and H3K36 demethylase Vitamin C. Fu et al. found that crotonic acid facilitated telomere rejuvenation through crotonylation and improved the generation of CiPSCs. Considering the association between epigenetic alteration and tumorigenesis, it may be necessary to explore if these types of compounds are safe or not in terms of oncogene activation or tumor suppression inactivation. Further, biophysical signals such as stiffness can also help achieve the direct reprogramming of somatic cells.

Furthermore, although chemical cocktails work safely on mice, re-evaluation is needed in human cell reprogramming due to the differences between mice and humans in epigenetic memories and different pluripotent signal pathways. Up till the present moment, even though mouse CiPSCs have advanced in the last several years, generation of human CiPSCs have not been reported yet, which means a large-scale screening of small molecules may be necessary. That said, a few cases of successful lineage reprogramming with pure chemical compounds, of human somatic cells have been reported. However, the risk and efficiency for chemical induction of human cells remains to be explored case by case for each particular clinical protocol.

### Table 2. Representative cocktails of chemicals applied in the generation of CiPSCs

| Source cell                  | Target cell | Small-molecule compound                              | Ref  |
|-----------------------------|-------------|------------------------------------------------------|------|
| Mouse Fibroblast            | CiPSC       | C6FZ (CHIR99021, 616452, FSK, DZNep) OR VC6TFZ (VAP, CHIR99021, 616452, Tranylcypromine, FSK, DZNep) | 4    |
| Mouse intestinal epithelial cells | CiPSC   | VC6TFE5Z (VAP, CHIR99021, 616452, Tranylcypromine, FSK, DZNep) + AM580 | 28   |
| Mouse neural stem cells     | CiPSC       | VC6TFE5Z (VAP, CHIR99021, 616452, Tranylcypromine, FSK, EPZ, Ch55, DZNep) | 28   |
3 Controlled mutagenesis of host gene caused by retroviral insertion and nanomaterial delivery system

Despite their robust efficiency, classical \(\gamma\)-retro- and lentiviral vectors-based gene transductions have been linked with random insertions into the host genome, which may lead to unexpected genomic modifications. Therefore, several DNA-free strategies have been developed to circumvent the random integration of transgenes into target cell genomes.

Sendai-virus (SeV), a non-integrating adenoviral vector, has been demonstrated to reduce the possibility of genomic modification or gene silencing and derive integration-free iPSCs effectively because it has a complete cytoplasmic replication cycle\(^{44,45}\). Other non-viral methods, such as recombinant protein transductions\(^{46,47}\), repeated transfection with modified mRNA, and microRNA (miRNA), have also been tested. For example, the direct transfection of mRNA or the lentiviral expression of ESC-specific miRNA, such as \(\text{mir302}\) and \(\text{mir367}\), can induce mouse and human somatic cells into iPSCs without introducing exogenous TFs\(^8\). These DNA-free methods proved that transgene insertion is not essential for iPSC production and that an efficient decrease of the genomic modification risk can be obtained. Specifically, the footprint-free iPSCs generated by this method do not involve permanent genomic alterations and can also be differentiated into desired cells. However, these technically challenging methods are shown to be less efficient than retroviral transduction. Furthermore, they can only reprogram rare cell types such as fibroblasts; currently, this method may not be desirable when used to produce clinical-grade cells. However, most methods still represent options for \textit{in vitro} biomedical applications, reducing the need for retroviral transduction\(^{49}\).

In addition, mRNA-based induction is a safe integration-free reprogramming method. However, due to the short half-life of mRNA and the obstruction of delivery, the efficiency of mRNA is lower than other methods\(^50\). Recently, self-replicating RNA (srRNA), an improved synthetic modified mRNA-based method was reported to be used in somatic reprogramming from human neonatal fibroblasts and was demonstrated to extend protein expression duration without risk for genomic integration. Steinle et al. believed that, due to the intergration-free property, single-shot
of srRNA with higher reprogramming efficiency will hold a good application prospect in reprogramming research field.\textsuperscript{51}

Moreover, delivery system is also one of important factors influencing the exogenous DNA integration. Although electroporation and chemicals are widely used, they may have the potential of reducing the cell activity or causing exogenous DNA integration. Nanomaterials may offer alternatives to traditional delivery methods because the scale measuring ten to hundred nanometer makes nanomaterials high and direct local stimuli.\textsuperscript{52} Wang et al. described a high-efficiency cellular reprogramming strategy by puncturing cells with an array of diamond nanoneedles.\textsuperscript{53} This strategy realizes the delivery of mini-intronic plasmid (MIP) to generate iPSC from human fibroblast. The delivery process is finished within 5 minutes without cell lift-off. The efficiency reaches to 1.17% ± 0.28% higher than traditional plasmid delivery methods. As alternative method, CRISPR/Cas9 system is usually delivered by plasmid, mRNA or ribonucleoprotein (RNP) complex, which has advantages of high efficiency and low off-target effect. However, RNP complex performs not well for cell reprogramming with multiple gene activation cell reprogramming. In contrast, magnetic molecularly imprinted polymers (MMIPs) carry multiple RNP and reach high efficiency.\textsuperscript{54}

4 Eliminating teratoma forming cells via the drug-inducible suicide system, small molecules and immunodepletion

iPSC heterogeneous somatic origin increases reprogramming uncertainty.\textsuperscript{22} Of note, when preparing cells for transplantation, the uncompleted reprogrammed cells, the undifferentiated iPSCs, and even the differentiated iPSCs may increase the oncogenic potential of therapeutic cells. Such observation is a consequence of the genetic or epigenetic aberrations from cellular reprogramming or prolonged cell culture.\textsuperscript{55, 56} Therefore, for a safe clinical application of iPSCs, it is essential to eliminate these cells during cell preparation. To this end, suicide gene technology is currently widely used to improve the safety of stem cell-based therapy. Specifically, the approach is to selectively eliminate aberrant therapeutic cells by activating a highly efficient safety switch. To date, three main suicide strategies have been developed.
One of these strategies is to use an anti-CD20 monoclonal antibody to induce antibody-dependent cytotoxicity, thus killing the cells expressing the B-specific human CD20 gene exogenously. For example, Inrona et al. achieved the elimination of CD3+CD20+ human T cells by adding monoclonal, chimeric anti-CD20 IgG1(kappa) Rituximab antibody (Roche) in the presence of complement. However, to date, no experiments have been performed applying CD20 to iPSCs field.

The second strategy is to use a metabolic suicide gene, herpes simplex virus thymidine kinase (HSV-TK) gene, and its prodrug, ganciclovir (GCV). The product of GCV yielded through phosphorylation by HSV-TK incorporates into replicating DNA, causing cell apoptosis. HSV-TK has been tested in human iPSCs as a suicide gene system. Studies have demonstrated that the HSV-TK-expressing cells can be eliminated both in vitro and in vivo with high specificity and efficiency. However, as Kimura et al. insisted, the HSV-TK system cannot sufficiently shrink the iPSC-derived teratomas in vivo. Furthermore, as a potent cytotoxic antiviral drug, GCV often unavoidably kills transplanted cells expressing the HSV-TK suicide gene system when used to treat herpes virus infections.

The most recent suicide gene system, inducible caspase-9 (iCASP9), highlights the potential of iPSC-based regenerative therapy with improved safety. Its operating principle is to substitute the caspase recruitment domain of pro-apoptotic caspase-9 with a mutated dimerizer drug-binding domain from the human FK506-binding protein (FKBP12-F36V). AP1903 (aka rimiducid), a chemical inducer, binds to the F36V mutation with a high affinity. Consequently, the dimerization of F36V, and the activation of caspase-9 and downstream effector caspases, such as caspase-3 and 7, occur (Fig.1). Therefore, upon adding AP1903 in the medium, the iCASP9 system can induce apoptosis. This system is effective in lentivirus infected T cells and human iPSCs. However, the nonspecific lentivirus-mediated genomic integration may lead to oncogenic, genetic changes, or unexpected silencing.

To overcome the random genomic integration, the iCASP9-based lentiviral vector’s genomic integration technique was improved to precisely introduce the gene into the genomic safe harbour.
AAVS1 locus. This resulted in the CAG promoter activating a strong and stable expression of iCASP9. The AAVS1 locus resides in intron 1 of the PPP1R12C gene on human chromosome 19; this locus is widely used as an ideal and stable genome safe harbour site. The small molecule AP1903 could cause iCASP9 dimerization and cell apoptosis, eliminating iPSCs and iPS-derived cells that integrated iCASP9. Of note, studies have shown that the iPSC-derived teratomas shrink dramatically upon applying AP1903.

As an improved application in the eliminating residual undifferentiated PCSs, Wu et al. selected the SOX2 locus as a safe harbor of the iCASP9 gene from three candidates, OCT4, Nanog and SOX2. Specifically, the SOX2 gene had a lower risk of an off-target effect than the other two loci. Compared with the AAVS1 locus system, the SOX2iCASP9 system can precisely eliminate the iPSCs without affecting iPSC-derived cells without SOX2 expression. However, the SOX2iCASP9 system cannot be used to eliminate undifferentiated iPSCs mixed with cell types expressing high levels of SOX2, such as neural progenitor cells and liver progenitor cells. In contrast with that, NANOG expresses in rare differentiated lineages and has been applied as safe harbor site by Martin et al. They engineered H9 hPSCs carrying three safeguard systems, NANOGiCasp9, ACTBTK and ACTBOiCasp9 and demonstrated their efficiency in ablating undesirable cell populations upon small molecule (AP20187 and/or AP21967) administration both in vitro and in vivo.

In addition, Lee et al. created another suicide system with a cytosine deaminase (CD) gene inserted within episomal vectors. CD converts non-toxic 5-FC (5-fluorocytosine) into 5-FU (5-fluorouracil), which can kill cells expressing the CD gene. Furthermore, the transduced episomal vectors with CD genes in cells may be lost following extended cell passaging. This suicide system gained exogenous DNA-free iPSCs and exogenous DNA-free neural stem cells. This combination of exogenous DNA-free vectors and suicide genes may have broad applications in the future.

To date, there are only small amount of alternative small-molecule-based suicide safety systems.
available for the research and clinical cell-based therapies. Specifically, iCASP9 suicide gene system has been demonstrated to be effective and safe in clinical trials. Table 3 summarizes the properties of suicide systems currently explored in iPSCs field. Considering the required long-term safety of iPSCs-based transplantation engrafted in human body, it is necessary to develop new systems of “keys” (ie, chemical inducers of dimerization, CID) and “locks” (ie, variations of the iCASP9-fusion protein) and evaluate the safety and efficacy of new combinations in clinical application of iPSC-derived cell products in the future.

Table 3. Current Suicide system applied in iPSCs field.

| Locks | Keys | Mechanism | Ref |
|-------|------|-----------|-----|
| Herpes simplex virus thymidine kinase (HSV-TK) | Ganciclovir | Inhibition of DNA elongation | 56, 58 |
| Inducible caspase-9 (iCASP9) | AP1903, AP21967, Ap20187 | Apoptosis induction | 6, 72, 73 |
| Cytosine deaminase (CD) gene | 5-flurocytosine | Inhibition of DNA elongation | 74 |
| CD-20 | Rituximab antibody (Roche) | Ag-Ab binding reaction | 54 |

In addition to the genetic methods, non-genetic means were applied to eliminating undifferentiated pluripotent cells. Chemical inhibitors of survivin, YM155, and cardiac glycosides, Digoxin and lanatoside C were reported to selectively ablate undifferentiated pluripotent cells with no damage to function and survival of differentiated cells. Immunodepletion is another effective strategy. Tang et al. used cocktail of antibodies against anti-stage-specific embryonic antigen (SSEA)-5 and pluripotency surface markers to remove teratoma-formation potential and obtain purified differentiated cell cultures. Most recently, the function of monoclonal antibody K312 and chimerised monoclonal antibody (mAb), ch2448, in depleting residual PSCS and prevented teratoma formation were reported. Of note, the premise of using this marker-based strategy is that the markers specifically expressed in PSCs, so as to avoid killing differentiated cells while removing undifferentiated PSCs.
5 Maximize the purity of iPSC samples

It is well known that fluorescence-activated cell sorting (FACS) and magnetically activated cell sorting (MACS) are used to efficiently isolate specific target cells from cell cultures.

Regularly, MASC is used to isolate iPSC-derived cells from derivation plates prior to transplantation. The main steps of MASC include labeling, loading, washing, and elution. Unlike somatic cells, iPSCs are usually passaged as clumps. Hence, it is necessary to obtain a single-cell suspension before the “labeling” step with specific antibodies. For example, Rho-associated protein kinase is used to dissociate iPSCs clumps (Fig.2). However, this technique has been proven to reduce the viability of iPSCs. More recently, Gao et al. adapted the DEF-CS medium to obtain single iPSC, achieving an over 80% viability rate \(^8^4\). Another problem requiring a solution is MACS’ limited efficiency in depleting undifferentiated iPSCs from a heterogeneous population of cells. Compared to the lower viability of target cells through FACS regarding negative selection, multiple magnetically labeled antibodies against the different surface antigens can improve the efficiency without affecting the viability. For example, TRA-1-60 or SSEA4 antibodies with MACS are useful for iPSC selection \(^8^5\)–\(^8^7\). As matter of fact, depending on the target cell type, specific surface markers have been successfully used for positive selection. For example, CD73+ photoreceptors have been isolated from iPSC-derived retinal organoids with high purity \(^8^8\). In a separate study, neural progenitor cells (NPCs) were separated from neural crest cells (NCCs) by MACS with CD271 depletion, followed by CD133 selection \(^8^9\). Highly efficient positive selection needs not only specific antigens but surface markers as well. It is therefore essential to select more specific surface markers on target cells to improve MACS efficiency.

Moreover, enrichment of label-free target cells from heterogeneous cell cultures is required for clinical treatment. Recently, a novel antibody-based beads, SpheriTech beads, are applied to purify target cells without label. The beads are paramagnetic and the affinity antibody is covalently immobilized onto their surface. Although the bead binding with cells are hold for washing in magnetic field as MACS, it is the label-free target cells that are eluted with trypsin and collected.
with high purification and activity. (Fig.2B). SpheriTech cell sorting has been compared with FACS and MACS to sort CD73 positive retinal photoreceptor progenitors from iPSCs induction with lower expense and more simplified operation than FACS and MACS.\textsuperscript{90} Besides, a responsive polymer-modified system could be option to be used to achieve label-free cells. Jiang et al. has used this system to separate label-free cell separation of iPSCs. Specifically, this system is based on the lower critical solution temperature (LCST) behavior of poly (di (ethylene glycol) methyl ether methacrylate) or PDEGMA, a thermo-responsive polymer used to make a homopolymer layer. For iPSC purification, the first step is incubating iPSCs at 37°C for five days to help them grow into cell colonies. Subsequently, the temperature is cooled to 22°C to promote the detachment of undifferentiated iPSCs from the layer, while differentiated cells remain attached (Fig.2). The latter approach leads to the separation of iPSCs from differentiated iPSC-derived cells, keeping the viability of iPSC-derived cells and the pluripotency of iPSC at high levels.\textsuperscript{91}

Except for antibody-based separation, specific chemical staining is available to sort iPSCs. Alkaline phosphatases (AP) with high expression in pluripotent stem cells, such as iPSCs, can hydrolyze phosphate in cells under alkaline conditions. Although AP staining is not a definitive standard for the established iPSC clones, the number of AP positive clones are applied to evaluate reprogramming efficiency.\textsuperscript{92} However, the AP colonies stained with previous substrate cannot be propagated any further. Recently, an improved substrate, AP Live Stain, is being used to measure and visualize the kinetic process of somatic reprogramming. The stained iPSCs colonies can be still further passaged and identified with additional specific markers because it does not change the characteristics and integrity of stained cells.\textsuperscript{87,93}

6. Assessment of the quality of iPSC before clinical use

It is realized that the safety and efficacy of iPSCs or iPSCs-derived cells must be evaluated before they can be used in clinical treatment. With the in-depth understanding of the proliferation and differentiation characteristics of iPSCs cells in vitro, researchers have put forward their own views regarding the quality control and related method.
Colter et al. noticed that the complexity of iPSCs production and downstream inductive differentiation process brought about uncertainty from processing to clinical effect, including low quality, heterogeneity and other problems. Therefore, the production and application of stem cells should rely on more rigorous and effective quality control of molecular and cell characteristics. It is necessary to establish a systematic evaluation of the variability between different batches of products and a complete datasets combining with relevant computational methods. These efforts will help to develop a practical model and improve the robustness of the production process.  

Assou et al. believes that, comparing with ESCs, iPSCs need additional mandatory quality control because of the possibly acquired genetics changes, such as aneuploidy and oncogene mutations (such as TP53), which directly affect the safety and efficacy of iPSCs and derivative products. Therefore, genome integrity testing should be used as a routine test item, and karyotype analysis should be a standard method for the evaluation. Therefore, it is logically reasonable that the mutation screening should be applied systematically and corresponding judgment standards should be established. Concerning the methods, short tandem repeats (STR) analysis can be considered as an essential indicator of genomic integrity in addition of karyotype. Indeed, STR analysis is also recommended as the mandatory item to exhibit the genomic integrity of iPSCs establishment. It is emphasized that the STR profile of qualified iPSCs should be established in early passages and must match the cell donor. The ANSI/ATCC ASN-0002-2011 standard for the authentication of human cell lines requires at least 8 core STR loci with an 80% threshold match. Kerrigan et al. increased the number of STR loci to 15 and Taylor believed 16 STR specific sites may be necessary for the plain identification even iPSCs are generated from autologous somatic cells. Besides, fluorescence in situ hybridization (FISH), array comparative genetic hybridization (aCGH), and other microarray approaches, such as quantitative PCR (qPCR), SNP arrays, digital drop PCR (DDPCR), and next generation sequencing (NGS) were also used to assess insertion and deletion (indel), copy number variables (CNV), and single nucleus variables (SNV). Baker et al. evaluated a set of CNV and SNV determination methods for the advantages and disadvantages. It is proposed that PCR technology and fish technology are more suitable for detecting known frequent small fragment mutations, while aCGH and NGS fit more for large fragment analysis.
However, when comprehensively considering cost, workload of data analysis and complexity of result interpretation, ddPCR is commonly applied at present, which has high accuracy and relatively low cost comparing with aCGH, NGS and FISH technologies. Therefore, when evaluating tumorigenicity of iPSCs and iPSCs-derived cells, the known oncogene mutations and choose targeted sequencing or ddPCR could be set as the top priority in analysis. Of course, because NGS is the whole genome at single base resolution and can detect most genetic anomalies, depending on the sequencing depth, it may be considered when a high standard is preferred. However, high depth NGS is difficult to become a routine mean of quality control before the sequencing cost is reduced to an affordable price.9,98,99

Rehakova et al. proposed a set of mandatory criteria and “for information only” tests, including differentiation, genetic stability, identity, vector clearance, morphology, pluripotency, robustness, viability and histocompatibility, and proposed a set of testing methods corresponding to the current good manufacturing practices (cGMPs) and regulations about production of clinical-grade iPSCs and ESCs lines 94. In addition, with regard to the detection of iPSCs heterogeneity, flow cytometry analysis is a reliable method because it is timesaving, and robust, and the results are quantitative and comparable among different laboratories. Baghbaderani et al. argued that for iPSC, the release criteria for clinical use should contain >70% of cells positive for SSEA4, OCT3/4, TRA-1-60, TRA-1-81 and <5% of cells negative for CD34+ 100. Recently, single cell RNA-seq shows its extreme power in elucidation of the heterogeneity of a cellular population101, exampled by hematopoietic stem cells and mesenchymal stem cells as we identified102,103, single cell RNA-seq may be applied in evaluation of the purity of the iPSC cells when a protocol is set up for a clinical product.

Besides the assessment items in vitro mentioned above, in vivo tumorigenicity are suggested to be included in the items, because cellular behavior in the engrafted site may be one of the most direct evidences to confirm the clinical usability of iPS cell therapy product (CTP). However, it is difficult to standardize the experimental conditions, such as the selection of animal model, the number of inoculated cells, the study duration and the site of transplantation. For instance, the immunocompromised mice are selected to test the tumorigenicity, but there is still not any
recognized standard on the number of animals and the controls which are required to demonstrate the CTP is unlikely to form a tumor. With regard to the site of transplantation, CTPs are now inoculated into the clinical equivalent site intended for patients via the clinical route, though its theoretical foundation is still debated. Moreover, the reduced lifespan of the test animal compared to humans also constitutes a limitation for the long-term observation of tumor formation\textsuperscript{104}. Therefore, Sato et al. pointed out that although customized assays have been established to test different products on a case-by-case basis, it is essential to reach a global consensus on the standard of the test approach so that it can be applied to any relevant CTP\textsuperscript{105}.

7 Conclusions

This review focused on current progress to diminish the tumorigenesis risk of iPSC technology, including reducing the potential of tumorigenicity and promoting the killing of abnormal cells, as shown in Fig. 3. This effort and further pursue outlined here are critical for the practical application of iPSC technology in its replacement therapy.

A reduction in the tumorigenicity potential of the produced iPSCs can be obtained by optimizing the cocktail of reprogramming factors, using the strategy of chemical inductive reprogramming, purifying cells by FACS and MACS, and applying exogenous DNA-free vectors. Although the traditional MACS purification strategy is firmly established, a wider variety iPSCs surface markers, apparatuses, and protocols remain to be explored, such as SpheriTech beads, a novel label-free affinity purification method. In addition, interdisciplinary contributions like polymer materials and nanotechnology have shown potential to separate iPS Cs and target cells, such as PDEGAM.

Currently, it is demonstrated that the suicide systems have very good application potential in eliminating the abnormal cells with teratogenicity in cell preparation from iPSCs effectively. However, for improvement of specificity and efficiency, future studies are needed to design and develop new "keys" and "locks" and select specific safeguard site for exogenous insertion.

Finally, systematic analysis of iPSC tumorigenicity, focusing on the mechanisms, is currently
lacking. However, it represents an essential characteristic of iPSCs technology. Therefore, the standard of evaluation for cell preparation tumorigenicity in clinical applications should be established in the future, in which the genetic integrity validation of iPSCs and derivations are essential. Specially, the assessment of the safety seems to be more essential when more and more effective techniques, such as CRISPR-dCas9 platform\textsuperscript{106,107}, are applied to fix the genetic defects of iPSCs derived from patients for the following therapy with the iPS CTP in clinic.

**Author contributions**

Chao liang Zhong and Miao Liu collected and organized the data and wrote the rough copy. Xinghua Pan and Haiying Zhu conceived the topic and revised the manuscript.

**Conflict of interests**

The authors declare that they have no conflict of interest. In addition, as an Associate Editor of *Precision Clinical Medicine*, the corresponding author Xinghua Pan was blinded from reviewing and making decision on this manuscript.

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Figure 1. Mechanism of iCASP9’s safety switch. The AAVS1 TALEN and the donor constructs are delivered to cells via plasmid transfection. Then, the antibiotic-resistant cells are selected. Next, the administration of AP1903 leads to the dimerization of FKBP12-F36V. As a result, caspase9 and the downstream effector caspases, such as caspase-3 and caspase-7, are activated, which lead to the cell apoptosis.
Figure 2. Schematic diagram of methods for the purity of iPSC samples. A. LCST behavior of PDEGMA. iPSCs are cultured on the polymer at 37°C for five days and grow into colonies on the layer. By cooling the temperature to 22°C, iPSCs colonies detach from the layer while differentiated cells remain on the layer. B. After incubation with SpheriTech beads coated affinity antibody, the target cells in heterogeneous cell suspension bind to beads. Label-free target cells are eluted from the bead by trypsin and affinity antibodies still stay attached to the beads. Comparing to SpheriTech beads, Antibodies or magnetic particles attaching to the target cells may influence the cells for positive selection of MACS. HCS: heterogeneous cell suspension; TCs: target cells.
Figure 3. Strategies for reducing tumorigenesis risk.