Cell Stem Cell

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Perspective

Pluripotent Stem Cell-Based Cell Therapy—Promise and Challenges

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SUMMARY

Human pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) provide unprecedented opportunities for cell therapies against intractable diseases and injuries. Both ESCs and iPSCs are already being used in clinical trials. However, we continue to encounter practical issues that limit their use, including their inherent properties of tumorigenicity, immunogenicity, and heterogeneity. Here, I review two decades of research aimed at overcoming these three difficulties.

INTRODUCTION

Pluripotent stem cells (PSCs) proliferate infinitely and differentiate into cells of all three germ layers. These two properties make PSCs attractive sources for cell therapies for various diseases and injuries. Two types of human PSCs (hPSCs) are being explored for clinical use: embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Human ESCs (hESCs) were first reported by James Thomson's group in 1998 (Thomson et al., 1998) seventeen years after the generation of mouse ESCs. This prolonged time lag between the creation of mouse and human ESCs was due to substantial differences in morphology and culture conditions, as described later. hESCs have been being explored in cell therapies for various diseases and injuries, such as spinal cord injury, age-related macular degeneration, and type 1 diabetes (Figure 1) (Ilic and Ogilvie, 2017).

With respect to clinical use, there are two concerns about hESCs: ethical issues regarding the usage of human embryos and immune rejection after transplantation. To overcome these issues, multiple groups have been trying to generate hESCs from a patient's own somatic cells by means of nuclear transfer. This strategy was popularized in 1996 by the creation of Dolly the sheep using nuclear transfer (Campbell et al., 1996). Although a report in 2004 fraudulently claimed the sussesful generation of nuclear transfer hESCs (Hwang et al., 2004), other groups have succeeded in producing these cells (Tachibana et al., 2013). The generation of human nuclear transfer ESCs remains technically challenging.

We took a different approach to the generation of pluripotent stem cells from somatic cells. We were encouraged by the success of somatic cloning in sheep, as well as by successful reprogramming by cell fusion between somatic cells and mouse ESCs (Tada et al., 2001). We hypothesized that pluripotency may be induced in somatic cells by defined factors that existed in ESCs and searched for reprogramming factors from mouse ESCs based on this hypothesis. We indeed identified four factors—Oct3/4, Sox2, Klf4, and cMyc—that were able to induce pluripotency in mouse fetal and adult fibroblasts (Takahashi and Yamanaka, 2006). We designated this new type of pluripotent stem cell as induced pluripotent stem cells (iPSCs).

Human iPSCs (hiPSCs) were generated in 2007 (Takahashi et al., 2007; Yu et al., 2007), and the progression from mouse to human iPSCs was accomplished in the brief period of one year due to accumulated knowledge from hESC research. Since then, many groups have been trying to bring iPSCs to patients, and some of them are already being tested in clinical trials (Figure 1)

Current expectations for realization of the promise of PSCs are at the highest they have ever been. However, there are many challenges that need to be addressed in order to bring PSC technology within the grasp of many more patients. In this Perspective, I would like to focus on the three major challenges: tumorigenicity, immunogenicity, and heterogeneity. I hope to expedite the development of cell therapies using hPSCs by discussing these challenges and providing potential solutions.

Tumorigenicity

An important advantage of PSCs is their potential for infinite proliferation, as a result of which we have been able to prepare billions of various types of human cells for transplantation. However, this property is a double-edged sword, because if cells keep proliferating even after transplantation, they may result in tumors. Three tumorigenic scenarios may be considered. First, if undifferentiated and/or immature cells are retained in the final cell products that have been differentiated from human PSCs, teratomas or tumors may emerge due to incorrect patterning. Second, if reprogramming factors remain active in the iPS cells, they may promote tumorigenesis. Third, tumorigenicity may be caused by genetic mutations that have occurred during *in vitro* culture of PSCs.

Teratoma and Other Tumors due to Incorrect Patterning

The formation of teratomas is the most serious problem for both hiPSC and hESC cell transplantation. Even a few residual PSCs could result in teratoma formation. In addition, if lineage-specific

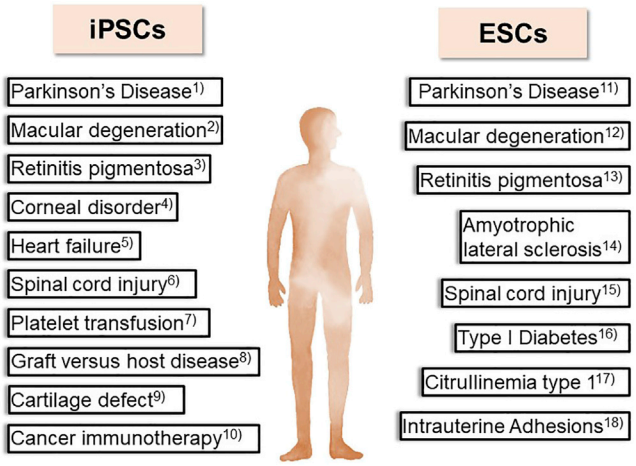

Figure 1. Clinical Trials for Cell Therapies Using PSCs

Shown are clinical trials that use hiPSC or hESC and are found in UMIN Clinical Trials Registry (https://www.umin.ac.jp/ctr/index.htm) or ClinicalTrials.gov (https://clinicaltrials.gov/ct2/home) as of Septermber, 2020. Web links are as follows:

(1) https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr_view.cgi?recptno=R000038278
(2) https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr_view.cgi?recptno=R000013279, https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr_view.cgi?recptno=R000029894, https://ClinicalTrials.gov/show/NCT04339764, https://ClinicalTrials.gov/show/NCT02464956
(3) https://jrct.niph.go.jp/en-latest-detail/jRCTa050200027
(4) https://jrct.niph.go.jp/en-latest-detail/jRCTa050190084
(5) https://jrct.niph.go.jp/en-latest-detail/jRCT2053190081, https://ClinicalTrials.gov/show/NCT04396899, https://ClinicalTrials.gov/show/NCT03763136
(6) https://jrct.niph.go.jp/en-latest-detail/jRCTa031190228
(7) https://jrct.niph.go.jp/en-latest-detail/jRCTa050190117
(8) https://ClinicalTrials.gov/show/NCT02923375
(9) https://jrct.niph.go.jp/en-latest-detail/jRCTa050190104
(10) https://ClinicalTrials.gov/show/NCT03407040, https://ClinicalTrials.gov/show/NCT04106167, https://ClinicalTrials.gov/show/NCT03841110, https://jrct.niph.go.jp/en-latest-detail/jRCT2033200116
(11) https://ClinicalTrials.gov/show/NCT03119636, https://ClinicalTrials.gov/show/NCT02452723
(12) https://ClinicalTrials.gov/show/NCT02286089, https://ClinicalTrials.gov/show/NCT03305029, https://ClinicalTrials.gov/show/NCT02590692, https://ClinicalTrials.gov/show/NCT01469832, https://ClinicalTrials.gov/show/NCT02941991, https://ClinicalTrials.gov/show/NCT03046407
(13) https://ClinicalTrials.gov/show/NCT03944239
(14) https://ClinicalTrials.gov/show/NCT03482050
(15) https://clinicalTrials.gov/show/NCT01217008, https://clinicalTrials.gov/show/NCT02302157
(16) https://ClinicalTrials.gov/show/NCT02239354
(17) https://dbcentre3.jmacct.med.or.jp/jmactr/App/JMACTRS06/JMACTRS06.aspx?seqno=9141
(18) https://ClinicalTrials.gov/show/NCT04232592

stem cells exist within the transplant, they may form tumors due to incorrect or incomplete patterning. For example, in the nervous system, patterning toward cortex produces a highly proliferative cell that can produce ''neural rosettes,'' which grow in a tumor-like fashion if injected *in vivo* (Malchenko et al., 2014). Therefore, researchers trying to initiate cell therapies with hPSCs have been spending considerable time and effort to devise methods that could prevent teratomas and other tumors from arising due to incorrect patterning.

The first step to reducing the risk of teratoma is to establish efficient methods of *in vitro* directed differentiation. Takahashi and colleagues achieved a purity of >95% in the first ever clinical transplantation effort where hiPSC-derived retinal pigmented epithelium was administered to patients with age-related macular degeneration, with few remaining cells being positive for markers of undifferentiated cells (Mandai et al., 2017). A relatively small number of cells were required, and this, along with the fact that the transplantion site was easily accessible, led to a clinical trial in 2014, just seven years after the first report of hiPSCs.

In contrast, in many other cases, more stringent purification procedures are needed to meet with the safety standards set for clinical trials. For instance, a clinical trial that is administering hiPSCs for Parkinson's disease is utilizing a method of positive selection of dopaminergic neurons with anti-Chorin antibody (Kikuchi et al., 2017). The development of good manufacturing practice (GMP)-compatible cell sorting systems enabled purification of mature differentiated cells with antibodies in their study. In another clinical trial for heart failure conducted by Sawa and colleagues in 2019, it was determined that CD30 was expressed in undifferentiated iPSC, but not in differentiated cardiac myocytes (Sougawa et al., 2018). To eliminate residual iPSCs, they utilized Brentuximab vedotin, an anti-CD30 antibody conjugated with the antimitotic agent monomethyl auristatin E, which has been approved for the treatment of CD30-positive lymphomas. In a clinical study for corneal epithelial stem cell exhaustion syndrome, Nishida and colleagues performed both negative and positive selections to purify corneal epithelial cells (Hayashi et al., 2017). They first removed undifferentiated hiPSCs and other undesired cells with antibodies recognizing CD200, which they found is an efficient negative marker for corneal epithelial cells (Hayashi et al., 2018). Following this negative selection, they performed positive selections with antibodies recognizing ITGB4 and SSEA-4.

In addition to residual PSCs, tumors may also arise from differentiated progeny that are still proliferative. This is an important issue in the clinical study for spinal cord injury, in which neural progenitor cells derived from hiPSCs (Figure 1) will be transplanted. Okano and colleagues have shown that this approach brought about functional recovery in rodent and monkey models of spinal cord injuries (Nakamura and Okano, 2013). However, with some hiPSC lines, paralysis reemerged due to proliferation of nestin-positive neural progenitor cells after transplantation, while such tumorigenicity was not seen with other hiPSC lines. Therefore, careful selection of iPSC lines is crucial for safe transplantation. In addition, the same group has devised another measure to suppress tumorigenicity of immature neural progenitor cells (Okubo et al., 2016). It has been shown that notch signaling is crucial for self-renewal of neural progenitor cells. By inhibiting this pathway with γ-secretase inhibitor, they succeeded in inhibiting expansion of immature neural progenitor cells. To further ensure safety, they plan to intentionally mismatch HLA alleles between recipients and transplanted hiPSCs in the initial clinical study so that transplanted cells can be eliminated by discontinuation of immunosuppressant if undesired cell proliferation is observed. They are also considering a suicide gene approach as an additional safeguard (Kojima et al., 2019).

Tumorigenicity Caused by Reprogramming Factors

This risk is specific for iPSCs. All of the four reprogramming factors have been associated with tumorigenicity, especially c-Myc, which is one of the most frequently mutated genes in human cancers and often functions as a driver mutation. Indeed, we have shown that chimeric mice made with iPSCs created by the induction of retrovirus-mediated transfection of the four reprogramming factors often developed tumors (Okita et al., 2007). We detected reactivation of c-Myc retrovirus in these tumors. Chimeric mice with iPSCs which had not been induced with c-Myc retrovirus did not show such tumors. In addition to the original four reprogramming factors, other factors are sometimes used, such as a dominant negative mutant of p53, to increase reprogramming efficiency (Hong et al., 2009). In iPSCs generated with the use of plasmids, EBNA1 is used to maintain episomal expression of the reprogramming factors (Okita et al., 2008). This is a risk, as the roles of EBNA1 in cancer is well documented. Thus one should be careful to rule out the integration of these cancer-causing transgenes in hiPSC slated for use in clinical cell therapies.

Tumorigenicity Caused by Genetic Abnormalities

This risk is common for hiPSCs, hESCs, and any other cells that are expanded *in vitro* prior to transplantation. Culture of the cells for *in vitro* expansion inevitably causes genetic alterations, such as chromosomal abnormality, copy number variation, and single nucleotide mutations. Traditionally, chromosomal abnormalities were monitored by karyotyping, and cells with abnormalities such as chromosomal deletion, duplication, or rearrangement are discarded for use in cell therapies and other downstream applications. In hESCs and hiPSCs, duplications of chromosomes 1, 12, 17, and 20 have been often seen after long-term expansion (Amps et al., 2011). PSC lines with such chromosomal abnormalities are excluded from cell therapy applications. In some cases, subcloning is performed to select cells devoid of abnormalities.

What is more difficult to evaluate, and can sometimes prove controversial, is smaller genetic alterations, such as single nucleotide variation (SNV) and copy number variation (CNV), which can be detected by next-generation sequencing and related technologies. Rouhani et al. showed that somatic cells have mutation rate of 14 SNVs per cell per generation, while iPSCs and ESCs exhibited a ten-fold lower rate (Rouhani et al., 2016). Nevertheless, hPSCs accumulate SNVs in cancer related genes, including the tumor suppressor gene TP53 (Merkle et al., 2017).

In the first clinical study for macular degeneration in 2014, we performed whole-genome sequencing (WGS) of iPSCs and retinal pigmented epithelial cells derived from hiPSCs upon the request of the Japanese government (Mandai et al., 2017). We did not detect non-synonymous SNVs, small insertions or deletions (Indels), or CNVs in ~600 cancer-related genes, as defined by COSMIC census list as well as a list generated by Pharmaceuticals and Medical Devices Agency (PMDA) (https://www.pmda.go.jp/files/000155730.pdf, in Japanese), and were thus granted permission from the government to perform the first in-human trial. Whole-genome sequence analyses were also required by the Japanese government in the clinical studies with allogenic retinal cells and corneal ephithelial cells. In sharp contrast, in the clinical trials for Parkinson's disease and heart failure, PMDA did not require WGS analyses. The reason behind this discrepancy is that WGS data cannot be easily applied to predict cancer risk of cells for two reasons: data acquisition and interpretation.

Despite the rapid progress of technology, it is still challenging to sequence significant portions of our genome, including repetitive elements. Cancer driving mutations in these repetitive regions will be overlooked. In addition, genetic changes with low allelic frequencies can easily be missed even in non-repetetive regions. Genetic alterations in PSCs can be classified into two types: those that exist prior to the derivation of cell lines and those that arise after the derivation. Allelic frequency of the former is either 50 or 100%. Most, if not all, of these changes can be detected by WGS. In contrast, allelic frequency of the latter is lower and can be easily overlooked. By changing the algorithms used in data analyses, these minor changes may be detected, but such conditions of detection have lowered stringency and would result in numerous false positives.

Interpretation is another important issue. If one detects a mutation in a cancer gene, the primary question is whether the given mutation substantially increases cancer risk and thus prevents usage of the PSCs in cell therapy. This is a very difficult question to answer. First of all, a consensus definition of cancer genes has not been established. COSMIC provides a list of genes that that have been causally implicated in cancer, but the genes listed in Census are constantly changing (Sondka et al., 2018). This constant fluctuation is a challenge from a GMP point of view. Second, not all non-synonymous mutations of cancer genes are pathogenic. For example, BRCA1 is one of the most famous cancer genes, and numerous mutations have been detected in BRCA1 gene through genetic testing (Peshkin et al., 2001). Many of them are thought to be benign and do not support prophylactic mastectomy. This classification has been a formidable task that requires multiple considerations such as family pedigree, conservation among species, and evidence in the literature (Eggington et al., 2014). Despite on-going efforts to predict a tumorigenic potential of a given mutation, many mutations in cancer genes are classified as "variance of unknown significance."

Another factor that complicates a clear risk analysis is that even healthy individuals have multiple mutations in cancer genes. Both germline and somatic mutations can occur, and the former is inherited from either sperm or egg and thus exists in all cells in the body. When compared with the standard human genome, any individual can possess non-synonymous rare mutations in ~50 cancer genes, which are not reported in SNP databases. Somatic mutations are ones that arise after fertilization. Of relevance to this discussion, recent studies have shown that even healthy individuals develop somatic mutations in multiple cancer genes in non-cancerous tissues such as facial skin (Martincorena et al., 2015) and esophagus (Yokoyama et al., 2019).

Two other issues further complicate interpretation of genome analyses. First, even synonymous mutations may be pathogenic (Supek et al., 2014). Single nucleotide mutations that do not change amino acid sequences are considered to be silent but may not be innocuous. Synonymous mutations could change DNA structure and functions, especially splicing, and potentially could alter encoded proteins. Second, mutations in intergenic regions may be pathogenic (Mosquera Orgueira et al., 2020). Non-coding regions occupy ~98% of genome and were called "junk" DNA. However, important roles have been assigned to those regions, such as binding to transcription factors and encoding non-coding RNAs. Thus, mutations in these regions could alter the transcription of cancer genes. Evaluation of

each mutation in non-coding regions would be an overwhelming task.

Even when well-established driver mutations are detected, there might be argument as to whether the mutation prevents cell therapy applications. For example, individuals with pathogenic mutations in BRCA1 have higher incidence of tumor formation in tissues like breast and ovary. Even with the mutation, tumors are not detected in childhood. Furthermore, there is no evidence that these individuals have higher risk of tumorigenecity in tissues like brain and heart (Dullens et al., 2020). Thus an important question is whether the same mutation affects therapeutic usage of pluripotent cells for varying diseases such as Parkinson's disease, spinal cord injury, and heart failure. This question merits further discussion.

Another critical issue is whether iPSC reprogramming is itself mutagenic. Gore et al. performed whole exome sequencing of human iPSC lines and identified ~10 non-synonymous novel SNVs in each clone (Gore et al., 2011). They argued that iPSC reprogramming is mutagenic. Similar results were reported by others (Ji et al., 2012). Another recent study by Araki et al., reported that reprogramming from fetal endothelial cells was less mutagenic because resultant iPSCs contained fewer numbers of novel SNVs (Araki et al., 2020). However, other studies attributed these "novel" SNVs to rare somatic mutations that pre-existed in the original cells prior to reprogramming (Young et al., 2012). Consistent with this, the number of SNVs increase with the age of donors (Lo Sardo et al., 2017). The result obtained by Araki et al. could be explained by age-dependent increase in somatic mutations in original cells. This controversy may arise from the difficulty in detecting very rare mutations in original somatic cells. To overcome this issue, Kwon et al. established iPSCs and clonally expanded fibroblasts from the same parental fibroblasts (Kwon et al., 2017). They observed comparable numbers of SNVs and indels between the sibling iPSCs and fibroblasts, suggesting that SNVs were largely attributable to rare mutations that had existed in the parental fibroblasts.

Immunogenicity

Immune rejection is another critical issue in cell therapy. iPSCs created from the patients' own cells provide an unprecedented opportunity to perform autologous transplantation with pluripotent stem cells. However, immunogenicity of autologous iPSCs has been controversial. In 2011, Zhao et al. reported that iPSCs derived from C57/BL6 (B6) mouse often failed to produce teratomas when subcutaneously transplanted into B6 mice (Zhao et al., 2011). In the case where teratomas did form, authors observed signs of rejection, such as T cell filtration. In contrast, B6-derived ESCs produced teratomas without T cell infiltration in B6 mice. The authors attributed this apparent immunogenicity of autologous iPSCs to abnormal gene expression. More recently, *de novo* mutations in mitochondria were proposed as a potential source of neoepitopes of autologous iPSCs (Deuse et al., 2019). However, immunogenicity of autologous iPSCs has not been supported by other publications. Araki et al. generated multiple ESC and iPSC lines from B6 mice and differentiated them into skin or bone marrow tissues (Araki et al., 2013). After transplanting these differentiated cells into B6 mice, they did not observe differences in engraftment or T cell infiltration between ESC s and iPSCs. Guha and colleagues also performed similar experiments and did not detected immunogenicity of syngeneic iPSC-derived differentiated cells *in vitro* and *in vivo* (Guha et al., 2013).

Autologous iPSCs and derived grafts may provide an ideal option for transplantion in terms of circumventing an immune response. In the first clinical study for age-related macular regeneration, evidence of transplanted cells was detected by imaging analyses more that 2 years after surgery, without obvious signs of rejection (Mandai et al., 2017). Thus it is now safe to say that autologous iPSCs do provide an opportunity for rejection-free cell therapy using PSCs. Another study suggests that autologous iPSC-derived retinal pigment epithelium transplantation results in improved vision in animal models of age-related macular degeneration, and it is currently being tested in humans (Sharma et al., 2019). However, at the present time, allograft approaches are preferred over autologous modalities due to considerations of cost of production both in terms of monetary value and time. In severe indications including heart failure and spinal cord injury, the extended time taken for production of clinical-grade autologous cell products would not be amenable for the successful treatment of these acute conditions.

Traditionally, rejection in allografts has been overcome by the use of immunosuppressants. In organ transplantation, patients need a life-long immunosuppression. Despite substantial progress in designing drug combinations and protocols, patients may still suffer from severe side effects, such as infections. However, in immune-privileged tissues, such as central nervous system (Carson et al., 2006) and eye (Taylor, 2016), the duration of immunosuppressant may be shortened in specific instances. In fact, long-term engraftment has been reported in a Parkinson's disease patient who received neural stem cells from aborted fetus 24 years ago (Li et al., 2016). In this patient, immunosuppressive treatment (prednisolone, azathioprine, and cyclosporine) was slowly tapered and then stopped 64 months after transplantation. Likewise, long-term engraftment for up to two decades have been reported in other Parkinson's disease patients in multiple studies (Li et al., 2016). Similarly, immunosuppressants are administered transiently in clinical trials related to spinal cord injuries, Parkinson's disease, and corneal epithelial stem cell exhaustion syndrome with hESCs or hiPSCs. However, immune privilege may be broken by damage, trauma, or disease and may be weakened with aging. Furthermore, in the case of transplantation in non-immune-privileged tissues, life-long immunosuppression may be required.

HLA Haplotype Banks of Human PSCs

Another method used to reduce rejection is the matching of HLA haplotypes, an approach which is currently and widely used in hematopoietic stem cell transplantation. Millions of donors are registered in world-wide bone marrow banks. Cord blood samples are also available for patients suffering from leukemia and other blood disorders. However, preparing hPSC lines with thousands of unique HLA haplotypes is not practical. Instead, Taylor et al. proposed that 10 hiPSC lines homozygous for common HLA types selected from 10,000 donors provided a complete HLA-A, HLA-B, and HLA-DR match for 37.7% of recipients and a beneficial match for 67.4% in UK population (Taylor et al., 2005) (Figure 2). The same strategy should work for other ethnic populations. The challenge is how to identify HLA homozygous embryos from those preserved in infertility clinics. With the advent of iPSC technology, this approach has become

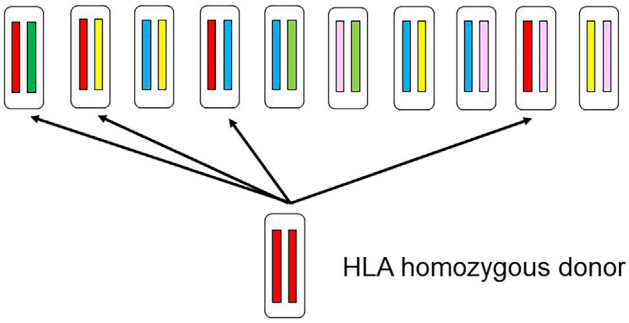

Figure 2. Advantage of HLA Homozygous Donors
HLA haplotypes of 10 individuals are shown with different color combinations (upper). None of them have an identical or ideal combination. iPSC lines from one HLA homozygous donor shown in lower panel would provide HLA matching to 4 out of 10 individuals shown in upper panel.

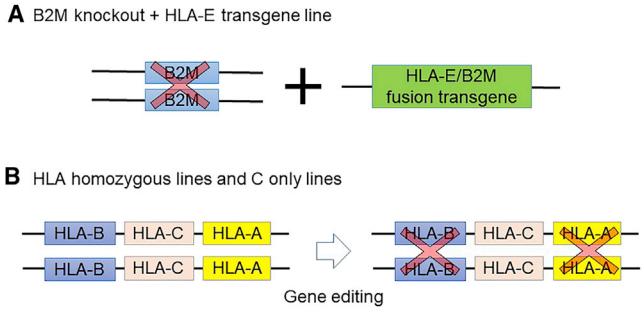

Figure 3. Two Approaches to Reduce Immunogenicity of Allografts
(A) Universal cell approach. All class I MHC molecules are inactivated by deletion of B2M gene. An HLA-E/B2M fusion transgene is then introduced into B2M null iPSC to prevent the cytotoxicity of endogenous NK cells.
(B) HLA homozygous iPSC lines and C-only approach. A small number of HLA homozygous iPSC lines can cover a large population. Disruption of HLA-A and HLA-B, leaving HLA-C, would cover wider population. HLA-C would function in the presentation of epitopes derived from tumors and viruses, if required. HLA-C would also suppress NK cell activation.

more feasible since iPSCs can be generated from donors whose HLA haplotypes have been determined (Nakatsuji et al., 2008). By collaborating with existing biobanks, such as bone marrow banks and cord blood banks, HLA homozygous donors can be effectively identified (Umekage et al., 2019). Once informed consent is obtained, blood samples may be collected from them for iPSC generation. In the case of cord blood banks, frozen blood samples in the bank can be used for iPSC generation. Rare haplotypes in one country or ethnic group may be covered by international collaboration.

The necessity for HLA-matching in order to prevent rejection varies with the end application. In monkey models, immune reactivity after transplantation of retinal pigmented epithelial cells was minimal when MHC alleles were matched (Sugita et al., 2016). This was confirmed in a clinical study in which HLA homozygous retinal cells were transplanted into HLA-matched patients (Sugita et al., 2020). In all five cases, survival of transplanted cells was observed one year later, without the systemic application of immunosuppressant. However, results of transplantation in brain are conflicting. Morizane et al. reported that MHC matching of iPSC-derived dopaminergic neuron transplants and recepient monkeys significantly reduced immune reactions and increased engraftment (Morizane et al., 2017). In contrast, Aron Badin et al. did not observe significant improvement in engraftment of neural cells in monkey brain as a result of MHC matching alone (Aron Badin et al., 2019). In cardiac myocyte transplantation, MHC matching did decrease immune response, but immunosuppression was also required to ensure engraftment (Kawamura et al., 2016). Thus even with HLA-matching, it appears that immunosuppression will still be required. Nevertheless, one can expect to reduce the dose and duration of immunosuppression by matching HLA, which would be a significant advantage for patients.

HLA Cloaking Approach

More recently, another approach has emerged with the advent of gene editing technologies, especially CRISPR technology (Lanza et al., 2019). HLA genes can be inactivated in pluripotent stem cells (Figure 3). All class I MHC, including HLA-A, HLA-B, and HLA-C, can be inactivated by deleting their common component beta 2-microglobluin (B2M) gene. Likewise, the expression of HLA-DP, HLA-DQ, and HLA-DR can be suppressed by deleting one of four transactivators essential for transcription of class II MHC genes, including CIITA (class II, major histocompatibility complex, transactivator). This approach is known as HLA cloaking. Lack of class I MHC, however, would result in lysis by natural killer (NK) cells, a phenomenon known as "missing self recognition" (Ichise et al., 2017). NK cells have multiple inhibitory receptors, such as NKG2A receptor and killer cell immunoglobulinlike receptors (KIRs), and activation of these receptors by ligands suppresses lysis activity. The class I MHC molecules functions as ligands of these receptors, and thus cells that express class I MHC are not attacked by NK cells. In contrast, when class I MHC molecules are cloaked, these cells are targeted by NK cells.

One strategy to overcome NK activation is to introduce a chimeric molecule consisting of HLA-E and B2M (Gornalusse et al., 2017). HLA-E is a non-classical MHC class I molecule with limited polymorphisms, which binds and activates NKG2A. Thus, an hESC or hiPSC line, in which B2M (and CIITA if necessary) is deleted and HLA-E-B2M has been introduced, could function as a universal cell line for all recipients (Figure 3A). Xu et al. proposed another strategy for HLA cloaking (Xu et al., 2019). They deleted both HLA-A alleles, both HLA-B alleles, and one HLA-C allele, leaving one intact HLA-C. It has been shown that among class I MHC molecules, HLA-A and HLA-B are critical for immune rejection. Thus, deletion of HLA-A and HLA-B alleles should suppress activation of CD8+ killer T cells. In contrast, the leftover HLA-C should bind KIR and suppress NK cells. If necessary, CIITA is also deleted to suppress class II MHC. Since HLA-C haplotypes are less diverse than HLA-A or HLA-B haplotypes, the authors estimate that as few as 10 lines with various HLA-C haplotypes can cover most of world's population. This C-only approach can be effectively generated by utilizing HLA-homozygous lines, since only two guide RNAs, one for HLA-A and another for HLA-B, are required (Figure 3B).

Haplotype Bank versus Cloaking

The two approaches, HLA haplotype bank and HLA cloaking, both have advantages and disadvantages. An HLA haplobank does not require genome editing. Despite the rapid progress in CRISPR technology, off-target effects are still a concern (Kosicki et al., 2018). Multiple rounds of gene modifications are accompanied by significant increase in cell divisions; thus, an accumulation of

somatic mutations is unavoidable. The presence of class I MHC cluster would support immunogenicity against tumor antigens and viral antigens. Thus, if the transplanted cells become tumorigenic or infected by viruses, these cells can be eliminated by CD8+ killer T cells. Presence of the intact class I MHC cluster, including HLA-C and HLA-E, is also advantageous in terms of suppressing NK cell activity.

However, it is noteworthy that HLA matching with homozygous donors does not necessarily guarantee privilege from NK cell attack. There are two groups of HLA-C, namely HLA-C1 and HLA-C2, where each binds to KIR2DL3 and KIR2DL1, respectively (Ichise et al., 2017). When HLA homozygous iPSC lines, either C1/C1 or C2/C2, is used for patients with C1/C2, missing-self activation of NK cells would occur. Since the allotype frequencies of C1 versus C2 in Japanese populations is 92.7:7.3, this mismatch would be rare. However, in other populations in which the frequency of C2 is higher, this issue would be more significant. For example, in Polish populations, the C1-C2 ration in 6:4 (Ichise et al., 2017). Nevertheless, the HLA matching approach is likely less sensitive to NK cell attack than is the HLA cloaking approach that relies only on a single ligand, such as HLA-C or HLA-E.

The biggest advantage of the HLA cloaking approach is the small number of lines required to cover the entire world population. It is possible that a single hiPSC line could serve as a universal source of cells. This is extremely attractive in terms of therapeutic production for various reasons. First of all, it is cost effective. At the moment, an expense of at least a couple hundred thousand dollars is required to generate a GMP-grade hESC or hiPSC line. In addition, regulatory authorities would demand rigorous pre-clinical data for each individual cell line. Furthermore, as discussed below, heterogeneity among cell lines is a hurdle for the usage of multiple lines for cell therapies.

Heterogeneity

PSCs share the same two properties: pluripotency and infinite proliferation. However, each PSC line is not identical to another. Each line is different in morphology, growth curve, gene expression, and propensity to differentiate into various cell lineages. This ''heterogeneity'' is a hurdle for downstream applications, including cell therapies.

Heterogeneity in Mouse PSCs

Heterogeneity was first recognized in mouse ESCs. It is well known that the ability of mESCs to make chimeric mice and undergo germline transmission is dependent upon the mouse species; only mESCs derived from 129 strains have this ability (Bradley et al., 1984). Accordingly, knockout mice of thousands of genes have been generated by using 129-derived ESCs. In contrast, ES cells from other mouse strains show poor chimerism and germline transmission. This suggests that genetic background seems to be the primary cause of heterogeneity of mouse ESCs. However, multiple ESC lines from the same 129 strain also show variation in their ability to make chimeras, suggesting a role for epigenetic factors. After each round of gene manipulation, one needs to select clones that can undergo germline transmission to establish a genetically modified mouse line. Thus, the combination of genetic variation and epigenetic modification are responsible for the heterogeneity of mouse ESC lines.

Smith and colleagues have shown that these inter- and intra-species heterogeneity of mouse ESCs can be neutralized by converting cells into the ''ground'' state. The conversion is achieved by treating cells with inhibitors of two kinases, the so-called 2i-treatment, namely MEK and GSK3 inhibitors (Ying et al., 2008). After 2i treatment, 129 ESCs achieve a ''ground state'' that is characterized by an undifferentiated morphology, lower DNA methylation content, and greater potential to efficiently produce chimeric mice and germline transmission. The 2i treatment enables generation of germline competent ESCs even from non-129 strains. This is likely due to erasure of epigenetic variation by the 2i treatment.

Heterogeneity in Human PSCs

Heterogeneity has also been reported in hESC lines. Osafune et al. examined 17 hESC lines for their differentiation potentials (Osafune et al., 2008). They detected >100 fold differences in the expression levels of lineage-specific genes among multiple lines. Due to these variations, some lines were optimal for pancreatic differentiation, while other lines were good for cardiomyocyte generation. Keller and colleagues also reported significant variations among mouse and human PSCs in their cardiac differentiation protocols (Kattman et al., 2011). Their protocol includes the use of bone morphogenetic protein 4 (BMP4) and activin/nodal for directed cardiac differentiation *in vitro*, and they observed that the concertation of these two growth factors had to be optimized for individual cell lines.

Heterogeneity is also an important issue with iPSCs. Earlier studies that compared limited number of hESC and hiPSC lines argued that there were significant differences in gene expression, epigenetic status, and differentiation potentials between two hPSC lines (Yamanaka, 2012). However, later studies using greater sample numbers argued against the earlier studies. Comparison of twenty or more hESC and hiPSC lines demonstrated that both ESCs and PSCs have overlapping variations. A carefully designed study by Hochedlinger and colleagues demonstrated that genetically matched hESCs and hiPSCs are indistinguishable (Choi et al., 2015). They showed that genetic background is the biggest factor in determining heterogeneity in gene expression. Another study demonstrated the importance of genetic background by measuring clonal differences in hepatic differentiation from hiPSCs (Kajiwara et al., 2012).

However, some iPSC lines do show defects in differentiation ability. In neural differentiation, most hiPSC lines were comparable to ESCs and differentiated into Pax6 positive cells with more than 95% efficiency (Koyanagi-Aoi et al., 2013). However, some iPSC lines showed ~80% neural differentiation efficiency, with appreciable numbers of residual undifferentiated cells. Transplantation of these cells into brains of immune-deficient mice resulted in teratoma formation. Abnormal expression of endogenous retroviruses, due to DNA hypomethylation, was detected in these tumorigenic clones. A similar result was obtained in experiments related to hematopoietic differentiation (Nishizawa et al., 2016). Some iPSC lines showed lower differentiation potential, accompanied by abnormal epigenetic status. Thus, epigenetic variations also contribute to heterogeneity.

Naive Human PSCs

To overcome heterogeneity, some researchers have attempted to convert a ''primed'' state of hPSCs into a ''naive'' state. In the conventional culture condition with fibroblast growth factor 2 (FGF2), hPSCs resemble postimplantation epiblast in gene expression and epigenetic status. This status is designated

primed. In contrast, mouse ESCs and iPSCs resemble inner cell mass of blastocyst or preimplantation epiblast. This state is designated naive. Primed mouse stem cells were also established from late epiblast and designated epiblast stem cells (EpiSCs), which resemble human ESC and iPSC in morphology, gene expression, and culture conditions (Brons et al., 2007, Tesar et al., 2007). The differentiation potential of EpiSCs is limited when compared to naive mouse ESCs and iPSCs in that they do not make chimeras when injected into mouse blastocyst. Induction of ground state by 2i inhibitors further enhance differentiation potential of mouse PSCs. These findings may suggest that differentiation capacity of hPSCs can be enhanced by converting them into the naive and ground states.

Multiple approaches have been reported as to how to induce naive or ground state pluripotency in hPSCs. One approach is to use a combination of chemical inhibitors of growth factors (Theunissen et al., 2014). In human, inhibitors of MEK and GSK3 plus LIF (2iL) alone did not induce naive conversion, but instead caused differentiation into neural lineage. However, by adding inhibitors of three kinases, ROCK, BRAF, and SRC, in the presence of activin and hLIF, Jaenisch and colleagues achieved conversion of human ESCs to the naive state (Theunissen et al., 2014). In an alternate protocol, Takashima et al. induced the naive state by overexpessing two transcription factors, NANOG and KLF2, in the presence of 2iL (Takashima et al., 2014). Once established, the naive state was maintained in the presence of a protein kinase C (PKC) inhibitor, without transgene expression. Naive human cells established by these two methods showed global DNA demethylation. Thus, using these procedures, heterogeneity caused by epigenetic variation may be nullified.

One concern about naive hPSCs is their genetic integrity. In the naive human cells generated by five kinase inhibitors, chromosomal abnormalities were repeatedly observed (Theunissen et al., 2014). In addition, Avior et al. reported that naive hESCs contained more SNVs than did primed counterparts (Avior et al., 2019). This may result from the increased rate of cell division of naive cells. Alternatively, pathways involved in DNA damage and repair may be downregulated in the human naive pluripotent state. More recently, another protocol using reduced concentration of the MEK inhibitor resulted in an increased rate of proliferation along with fewer chromosomal abnormalities (Di Stefano et al., 2018). Further studies are required to understand this important issue. Additional intermediate states of pluripotency continue to be described (Cornacchia et al., 2019), with potential implications to the above discussion.

Another concern about naive hPSC is loss of imprinting. As mentioned above, one characteristic feature of naive hPSC is global hypomethylation. Upon re-differentiation back into primed state, most genomic regions are re-methylated. This is not the case, however, for imprinted genes. Most imprinted patterns remain erased in re-primed cells (Theunissen et al., 2016). Abnormal imprinting may hinder clinical applications of naive hPSCs.

CONCLUSION

The potential of human pluripotent stem cells in cell therapies and other applications is enormous. Cell therapies for more than 14 diseases and injuries have reached or are about to reach clinical trials (Figure 1). More sophisticated applications of hPSC technology are also making steady progress: these include the differentiation of hematopoietic stem cells from PCSs for leukemia and other blood disorders, creation of liver organoids for treating liver failure, and similarly, creation of kidney organoids for kidney failure. While I have discussed the major challenges to bringing hPSC-derived products to the patient in this Perspective, there have been several encouraging success stories to spur us into activity and hundreds of scientists are continuing to work with great skill to overcome the remaining hurdles. For example, sensitive *in vitro* systems, such as organ-on-a-chip models, are being developed to predict tumorigenicity (Sato et al., 2019), and factors that can reduce heterogeneiety have been identified (Kunitomi et al., 2016). I am confident that we can bring PCS technologies as a viable option for the treatment of patients globally, in the not-so-distant future.

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DECLARATION OF INTERESTS

S.Y. is an inventor of patents about iPSC technology. S.Y. is a scientific advisor, without salary, to iPS Academia Japan Inc., which handles licenses of the patents.

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