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PERSPECTIVE

The future of iPS cells in advancing regenerative medicine

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Summary

Induced pluripotent stem (iPS) cells have great potential in regenerative medicine, including cell replacement therapies and disease modelling *in vitro*. However, with this potential comes several challenges, including clinical safety, reprogramming and differentiation efficiency, and compromised functionality of differentiated cell types after transplantation. Many of these issues arise from imprecise control of cell fate. With large-scale sequencing and genome-editing technologies we can now precisely manipulate the genome, which has expanded our knowledge of functional cell types and cell identity. These technologies may improve our efforts in generating iPS-derived therapeutic cells and in development of therapies for human diseases.

1. iPS cells and their potential applications in regenerative medicine

(i) *Generation and advantages of iPS cells*

Pluripotent stem cells are of great value in both scientific studies and clinical applications. They can differentiate into any cell type in the human body and, thus, hold tremendous promise in regenerative medicine. Before 2006, pluripotent stem cells could only be derived from blastocysts and early embryos (called embryonic stem [ES] cells), which could barely meet the great needs of scientists, clinicians and patients. Additionally, many ethical issues surround the use of ES cells for research. The generation of induced pluripotent stem (iPS) cells in mice in 2006 (Takahashi & Yamanaka, 2006) and in humans in 2007 (Takahashi *et al.*, 2007) fundamentally changed our views about stem cells in research. These cells are nearly epigenetically identical to ES cells, and they can functionally self-renew and differentiate into any cell type in the body.

Compared to ES cells, iPS cells have several attractive advantages. First, iPS cells are immunologically compatible, which makes them more feasible for regenerative medicine (Nishikawa *et al.*, 2008). Clinically, immunologic rejection is always a problem during tissue/organ transplantation. By deriving the desired cells from the patient's own iPS cells and transplanting them into the same patient, we can avoid immunologic rejection. This approach could bring the dream of personalized therapy to fruition. Second, iPS cells generated from cells of patients with genetic diseases, such as Alzheimer's disease and thalassemia, can be used as *in vitro* disease models for pathological research and drug screening. However, there is a limited supply of diseased embryos that can be used to generate human ES cells for similar studies. Third, iPS cells can be generated from almost all types of cells of the body because of the methodological improvement, especially from small molecule applications (Li *et al.*, 2013; Zhang *et al.*, 2015), so they are much easier to obtain than ES cells. Finally, iPS cells have less ethical issues surrounding them.

(ii) *Potential applications of iPS cells*

iPS cells have two potential applications that have captured the most attention in regenerative medicine: cell replacement therapy and disease modelling *in vitro*. iPS cells can be differentiated into any desired cell type and then transplanted to replace damaged or diseased cells, which makes them an excellent resource for cell replacement therapy. Alipio *et al.,* differentiated mouse iPS cells into pancreatic beta-like cells, which are similar to their physiological counterparts (Alipio *et al.*, 2010). These beta-like cells

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secreted insulin in response to glucose stimulation and, after transplantation, they corrected the hyperglycemic phenotype in two mouse models of type 1 and type 2 diabetes. These results support that iPS cells derived from somatic cells could effectively treat type 1 or type 2 diabetes. Additionally, Takahashi's group transplanted autologous sheets of iPS-differentiated retinal pigmented epithelial (iPS-RPE) cells from nonhuman primates back into their host and showed that the hosts did not display immune rejection or tumor formation (Kamao *et al.*, 2014). More recently, Takahashi's group transplanted human iPS-RPE cells back into a patient with age-related macular degeneration that caused severe visual impairment (Garber, 2015). We are still awaiting the progress of this pioneering work; however, so far, this work has paved the way for iPS cells in cell replacement therapy in humans, which is a big step forward in its clinical application.

Another great application of iPS cells is for disease modelling *in vitro*. Reprogramming methods have significantly improved, especially with non-integrating vectors, mRNA, miRNA or recombinant proteins, which has eased the process of generating patient iPS cells bearing specific mutations and improved the safety of the cells. These mutated cells are a great resource for studying disease, screening drugs and testing toxicity. Many researchers have used iPS cells to model a variety of diseases, including hematopoietic diseases (Park *et al.*, 2008; Raya *et al.*, 2009; Ye *et al.*, 2009), neurological diseases (Dimos *et al.*, 2008; Kazuki *et al.*, 2010; Pedrosa *et al.*, 2011) and cardiovascular diseases (Narsinh *et al.*, 2011; Zhang *et al.*, 2011). These researchers created these models by differentiating patient-derived iPS cells with tissue-specific protocols to mimic disease phenotypes in *vitro*. Importantly, iPS cells can be generated from almost any individual of any genetic background and then differentiated into various cell types, highlighting their potential for personalized medicine.

2. Challenges of iPS cells in regenerative medicine

While iPS cells have proved valuable in research, they still create enormous challenges for researchers and clinicians who face issues of compromised functionality of differentiated cells, safety, time cost, financial burden, regulatory policy and more (Cherry & Daley, 2013). Currently, the bottleneck in moving iPS cells to clinical applications lies in generating high-quality therapeutic cells that function similarly *in vitro* and *in vivo*. Here, we will mainly discuss the problems that compromise the functionality of iPS-differentiated cells.

The compromised functionality in cellular reprogramming has complex causes. Every step during differentiation – including differentiation conditions, *in vitro* selection, initial rate of survival, long-term engraftment and communication with the niche – can impact functionality. Retinal pigmented epithelial (RPE) cells were chosen for the first clinical trial in humans (Garber, 2015; Jha & Bharti, 2015) because RPE sheets are relatively easy to obtain by differentiating ES or iPS cells in dishes. However, whether these RPE sheets can communicate with the niche and function like its physiological counterparts is still unknown. Nonetheless, these pioneering works suggest that the initial differentiation conditions critically affect cell survival and function after transplantation. Unfortunately, for many other cell types, we do not yet have reliable and efficient protocols – partly due to variability between labs and individual scientists – to differentiate iPS cells *in vitro*.

A key factor that contributes to compromised functionality is the imprecision in controlling cell fate (Dimmeler *et al.*, 2014). Cell fate is determined by chromatin status, which stores all information about a cell's identity, such as transcription factor binding, DNA and histone modifications, and the 3D organization of chromatin. All of this information determines how the cell functions and communicates with its surrounding cells. Thus, by improving the precision in controlling cell fate, we can enhance cell functionality. To this end, we must 1) generate cell identity blueprints with multiple layers of information, including transcriptomes, epigenomes and the 3D organization of chromatin; and 2) create corresponding blueprints in therapeutics derived from iPS cells. Fortunately, with new technologies, we are moving fast towards this goal.

3. New technologies put iPS cells in the spotlight

In recent years, we have seen a number of new technologies rapidly develop in the life sciences. Among them, next-generation sequencing (NGS) and clustered regularly interspaced short palindromic repeats (CRISPR) technologies have revolutionized how we probe the molecular mechanisms of human development and disease. NGS can provide the complete whole-genome sequence and more comprehensive genome-wide information at high resolution. CRISPR technology, however, is a powerful tool to precisely change the genome by either permanently deleting/inserting specific genetic sequences or temporarily adding/removing epigenetic information.

(i) *NGS and characterization of cell identity*

Contrary to conventional Sanger sequencing, NGS can generate high-throughput data at a lower cost and within a much shorter time frame, which has

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facilitated its rapid application in many different genome-wide studies. NGS provides a basic layer of information that includes a complete genetic sequence of our genome. To build on that layer, we can combine NGS with other methods to decipher different aspects of the genome (Shin *et al.*, 2014; Yang *et al.*, 2015). For example, RNA-sequencing can generate the whole transcriptome, chromatin-immunoprecipitation (ChIP)-sequencing or bisulfite-sequencing can create epigenomic maps (epigenomes), and combining chromatin conformation capture (e.g. 4C, 5C and Hi-C) with NGS can reveal the 3D organization of chromatin. By combining all of these methodologies in a targeted cell, we can generate a multi-layer cell identity (ML-ID) blueprint for each type of cell of therapeutic interest.

The first layer of information obtained for this ML-ID blueprint is the transcriptome. Before large-scale sequencing, we primarily characterized cell identity by one or multiple highly expressed transcription factor markers. For instance, MyoD is the marker for myogenesis (Lassar *et al.*, 1986), and Oct4 and Sox2 are widely accepted as pluripotent markers (Rosner *et al.*, 1990; Scholer *et al.*, 1990; Yuan *et al.*, 1995). The transcriptome encompasses all of the transcriptive information in a therapeutic cell type, so it can more accurately define and characterize cell identity.

The second layer of information features epigenomes, or genome-wide epigenetic modifications of DNA and histones, including DNA methylation, histone H3K4 methylation, H3K9 methylation, H3K27 acetylation and many others. Specific modifications often mark functional elements in the genome. Usually, the epigenetic status is closely correlated with the transcription state, so these epigenomes feature an in-depth explanation for the transcriptome. However, they can also reveal novel regulatory mechanisms. For example, the *Sox2* gene is a pleiotropic gene that is expressed in multiple cell types, including pluripotent stem cells and neural progenitor cells (Sarkar & Hochedlinger, 2013); however, a recent study showed that in these cell types, different enhancers uniquely activate its transcription (Phillips-Cremins *et al.*, 2013), suggesting that the epigenetics at enhancers may also be key to a specific cell identity.

Another layer of information for the ML-ID blueprint is the 3D organization of chromatin. Chromatins are not randomly distributed in the nucleus (Misteli, 2007); they are arranged in specific 3D structures with the help of other proteins, including CCCTC-binding factor (CTCF) as well as cohesins and mediators (Splinter *et al.*, 2006; Hadjur *et al.*, 2009; Kagey *et al.*, 2010). This organization seems to be consistent at the megabase scale between mammalian cell types and conserved across species. However, with 5C at a resolution of 4 kb, researchers found that a cell-type-specific chromatin organization could occur at the submegabase scale (Phillips-Cremins *et al.*, 2013). They also showed that while both ES cells and neural progenitor cells highly express Sox2 according to the transcriptome data, the two cell types demonstrate different chromatin organization around this gene. This shows that the differences in 3D chromatin organization can reveal cell-type-specific features that the transcriptome cannot. However, we still do not understand how these 3D features are correlated with cell functionality.

There are many other layers that can be included in the ML-ID blueprint. These layers include, but are not limited to, DNase-seq, which detects genome-wide open chromatin, MNase-seq, which detects nucleosome positioning, and hMeDIP, which detects the genomic distribution of a novel DNA modification, hydroxylmethylation (Tahiliani *et al.*, 2009; Song *et al.*, 2011). These NGS-based methods have been excellently reviewed elsewhere (Shin *et al.*, 2014; Yang *et al.*, 2015). In the future, as we build more layers into this blueprint, we will be able to more accurately characterize cell identity.

The corresponding ML-ID blueprint serves as a gold standard when generating specific types of therapeutic cells *in vitro*. At present, the first layer (transcriptome) has been widely used in studies to characterize the cell identity and evaluate the quality, but it seems that this is far from enough because of the small number of successful cases that have been translated into clinics. Characterization of cell identity with more layers of information is required. Because of the lack of successful clinical cases, we still do not know to what extent the characterization should be made for the generated therapeutic cells at this stage, and further investigations are needed. Meanwhile, the mouse model provides a valuable tool to evaluate the functionality of the generated cells *in vivo*. Cells that both meet the *in vitro* standard and pass the *in vivo* tests are considered safe and suitable for clinical use.

(ii) *CRISPR and conversion of cell fate*

CRISPR technology is one of the most exciting bioengineering discoveries of the last few years. It is a powerful method used to edit the genome in most model systems, including worms, fruitflies, mice and humans. In this technology, the Cas9 protein, guided by a 20-mer oligo, cuts a specific sequence in the genome to create different mutations (Jinek *et al.*, 2012). Conventionally, CRISPR has been used to modify the genome, including insertion and deletion (Indel), gene knock-in and gene knockout modifications. However, since its discovery, CRISPR has been modified to support a diverse array of applications. Most interestingly, two other variants, CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa), precisely manipulate

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the transcription or epigenetic state of almost any genomic site (Maeder *et al.*, 2013; Perez-Pinera *et al.*, 2013; Qi *et al.*, 2013). CRISPRa is a more feasible tool for endogenous gene activation than other engineered transcription factors, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). In this article, we will focus on the regenerative application of CRISPRa in the conversion of cell fate.

To manipulate cell fate, CRISPRa has the advantage of precisely activating a genomic site, either in gene body regions or non-coding regulatory regions. The CRISPRa system targets a precise 20 bp DNA sequence and, thus, can manipulate the activeness of a precise site in the genome. Recently, several labs have activated endogenous genes by precisely targeting their promoters in different cell lines with promising results (Tanenbaum *et al.*, 2014; Chavez *et al.*, 2015; Konermann *et al.*, 2015). Some studies have also shown that CRISPRa can convert cell fate, either from pluripotent stem cells (Kearns *et al.*, 2014) or a differentiated cell type (Chakraborty *et al.*, 2014). As two groups recently reported, super enhancers or stretched enhancers can mark specific cell identities (Parker *et al.*, 2013; Whyte *et al.*, 2013). We still do not know if these specific cell identities can be achieved by the epigenetic manipulation of these non-coding enhancer regions. To test that, CRISPRa would be a perfect tool.

CRISPRa can be easily scaled-up to generate libraries for large-scale screening of genomic elements of interests, especially important non-coding regulatory elements involved in cell fate commitment. Several studies have successfully used the conventional CRISPR system to find functional genes in specific biological processes (Gilbert *et al.*, 2014); however, no non-coding regulatory libraries have been reported yet. Primarily, enhancers may be good candidates for library preparation because they are relatively easy to identify in the genome due to their enrichment of specific epigenetic modifications that can be revealed by the ChIP-seq method. Previous studies have also shown that CRISPRa can activate the enhancer of *Oct4* to promote iPS cell generation in combination with overexpressed factors in mice (Gao *et al.*, 2014). It would be very interesting to test, at the genome scale, if these non-coding regulatory elements are involved in cell fate commitment, including the generation of iPS cells.

However, we should also be aware of the potential pitfalls of the CRISPR technology, as they may affect its application in clinical practice. These pitfalls have been extensively discussed elsewhere (Peng *et al.*, 2015). Generally, the off-target effect is the major concern. It may cause unpredicted genome editing, which poses potential risks for either basic scientific studies or clinical use. Difficulty in Cas9 delivery may also hinder its widespread application because it leads to a low efficiency of the final editing event. Besides, many other aspects may also affect its efficiency and specificity, and these include Cas9 activity, sgRNA design, target site selection, etc. Many studies have been carried out to improve both CRISPR specificity and efficiency. By mutating sgRNAs, the Zhang lab found that single-base mismatch up to 9 bp 5′ of sgRNA completely abolished genomic cleavage by Cas9 (Cong *et al.*, 2013). More recently, they also identified a smaller Cas9 from *Staphylococcus aureus* (SaCas9), which would be easier to deliver (Ran *et al.*, 2015). Further optimization and improvement of Cas9-based tools for genome editing will make the CRISPR technology more powerful and promising in future clinical practice.

Precisely controlling cell fate is essential for cell functionality. By using the ML-ID blueprint as a reference and CRISPRa as an editing tool, potentially combined with more conventional approaches, we could finely manipulate the transcriptome or epigenome to make iPS-derived therapeutic cells more similar to *in vivo* cells. These efforts would improve our ability to generate functional cell types for cell replacement therapy.

4. Closing remarks

iPS cells hold great promise in regenerative medicine, for both cell replacement therapy and disease modelling *in vitro*. To bring this promise to fruition we must understand cell identity and cell fate commitment. Much effort has already focused on improving the efficiency and safety of reprogramming and differentiation. However, we still must overcome the hurdle of compromised functionality of iPS-derived therapeutic cells, which largely arises from imprecisely controlling cell fate, either transcriptionally or epigenetically. Fortunately, with NGS technology, we can create a detailed cell identity blueprint depicting multiple layers of information for a specific cell type. Meanwhile, CRISPRa provides a valuable tool to precisely manipulate the chromatin status of iPS-derived therapeutic cells to mimic their physiological counterparts. These technologies will brighten the future of applying iPS cells in regenerative medicine and disease modelling.

Declaration of interest

None.

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