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Gene correction in patient-specific iPSCs for therapy development and disease modeling

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Abstract

The discovery that mature cells can be reprogrammed to become pluripotent and the development of engineered endonucleases for enhancing genome editing are two of the most exciting and impactful technology advances in modern medicine and science. Human pluripotent stem cells have the potential to establish new model systems for studying human developmental biology and disease mechanisms. Gene correction in patient-specific iPSCs can also provide a novel source for autologous cell therapy. Although historically challenging, precise genome editing in human iPSCs is becoming more feasible with the development of new genome-editing tools, including ZFNs, TALENs, and CRISPR. iPSCs derived from patients of a variety of diseases have been edited to correct disease-associated mutations and to generate isogenic cell lines. After directed differentiation, many of the corrected iPSCs showed restored functionality and demonstrated their potential in cell replacement therapy. Genome-wide analyses of gene-corrected iPSCs have collectively demonstrated a high fidelity of the engineered endonucleases. Remaining challenges in clinical translation of these technologies include maintaining genome integrity of the iPSC clones and the differentiated cells. Given the rapid advances in genome-editing technologies, gene correction is no longer the bottleneck in developing iPSC-based gene and cell therapies; generating functional and transplantable cell types from iPSCs remains the biggest challenge needing to be addressed by the research field.

Introduction

Human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have the developmental potential to give rise to all adult cell types and serve as important tools for studying human developmental biology, where animal models have significant limitations. Because patient-specific iPSCs carry the exact genetic information of the donor cells, they offer unprecedented opportunities to study disease mechanisms. Beyond their utilities in mechanistic research, human pluripotent stem cells can also serve as great alternatives to

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adult stem cells for developing novel regenerative medicine because of their capability to greatly self-renew while maintaining developmental potentials. The development of iPSC technology, which allows the generation of various functional cell types from a patient's own somatic cells, can potentially solve the immunological incompatibility problem that ESC-based therapies could face. One of the major hurdles in developing iPSC-based autologous cell therapy, however, is that in many cases, the patient-iPSCs still carry the genetic defects that are associated with the patients' medical conditions. In diseases, such as sickle cell disease, β-thalassemia and Duchenne muscular dystrophy, underlying genetic defects in hemoglobin, or dystrophin genes, would prevent the direct use of patients' own cells for regenerative therapy without genetic modification. The ability to genetically correct the disease-causing mutation(s) is, therefore, essential for developing iPSC-based autologous cell replacement therapy. Here, we review the history and current status of genome editing in human iPSCs, with a focus on genetic corrections of disease-related genes for developing cell and gene therapies.

History of human iPSCs and challenges of iPSC-based regenerative medicine

This year (2016) marks the tenth anniversary of Shinya Yamanaka's discovery of induced pluripotent stem cells (iPSCs) (Takahashi et al. 2007; Takahashi and Yamanaka 2006), which is without a doubt one of the most exciting scientific breakthroughs in recent history. As with embryonic stem cells (ESCs), iPSCs can be maintained in laboratories without compromising their pluripotency, the potential to give rise to all cell types found in a typical human body. Unlike ESCs, which are derived from the inner cell mass of blastocysts (Thomson et al. 1998), iPSCs are generated by transient ectopic expression of defined transcription factors in differentiated cell types. Because their generation does not involve disruption of human embryos, there are considerably fewer ethical concerns using iPSCs than using ESCs for research and development. The Nobel-winning discovery of iPSCs brought high hopes for millions of patients suffering from degenerative diseases. Within a few years after the discovery, patient-specific iPSCs have been successfully used in studies of disease modeling and drug screening (Stadtfeld and Hochedlinger 2010; Wu and Hochedlinger 2011). However, there were three biggest challenges to be overcome before the therapeutic and research potentials of iPSCs can be fully realized: (1) generating and maintaining safe iPSCs with high efficiency. (2) Producing functional cell types for both cell therapy and drug development. (3) Genetically modifying iPSCs using precise genome editing.

Human iPSC generation has undergone significant improvements since the first proof-of-concept studies (Park et al. 2008; Takahashi et al. 2007; Yu et al. 2007). Retroviral transduction has been gradually replaced by other integration-free methods, including episomal plasmid, mRNA transfections, and Sendai virus-based reprogramming (Takahashi and Yamanaka 2016). Somatic cell types used for reprogramming also expanded from the original skin fibroblasts to many different cell types (Aasen et al. 2008; Eminli et al. 2009; Giorgetti et al. 2009; Haase et al. 2009; Kim et al. 2009; Liu et al. 2010; Loh et al. 2009; Sugii et al. 2010), with peripheral blood being an increasingly popular choice because of the

ease of sample collection, processing, and the amenability to episomal vector-based reprogramming (Chou et al. 2011). Protocols for generating and maintaining cGMP-complaint human iPSCs have also been developed and are expanding. Currently, generating patient-specific iPSCs that possess safety profiles similar to that of ESCs can be routinely achieved, and is no longer a significant roadblock. The major remaining issue related to reprogramming is the “naïve” vs. “primed” pluripotency (Gafni et al. 2013; Hanna et al. 2010; Takashima et al. 2014; Theunissen et al. 2014; Ware et al. 2014). Although delineating the similarities and differences between these two pluripotent states would have a significant value in understanding epigenetic regulations of pluripotency, there is not yet enough evidence to suggest that either type of the human iPSCs is superior in their differentiation efficiency or is safer in developing regenerative medicine. Much of the recent effort in the stem cell field has been devoted to genetic engineering and functional differentiation of human iPSCs.

Development of genome-editing technologies in human pluripotent stem cells

Homologous recombination (HR) in mouse ESCs, a now standard procedure in generating genetic mouse models, has revolutionized studies of mammalian genetics and developmental biology (Capecchi 2005; Doetschman et al. 1987; Thomas and Capecchi 1987). Since the derivation of the first human ESC lines (Thomson et al. 1998), it has been anticipated that similar technologies can be translated into human cells to uncover human-specific knowledge and to develop novel therapies. Replicating the gene-targeting success in human pluripotent stem cell system, however, was proven to be challenging and few laboratories succeeded before the recent advancement in engineered endonucleases. Lower single-cell survival and clonal efficiencies of human ESCs than that of murine ESCs and their fundamental differences in intrinsic cellular machineries governing DNA repair and genomic integrity are all significant contributors to the low success rate of human stem cell gene targeting. The first success of HR-based gene targeting in human ESCs was described by the Thomson laboratory 5 years after their original report of human ESC derivation (Zwaka and Thomson 2003). Although a significant achievement, the observed gene-targeting efficiencies are substantially lower than those achieved in mouse ESCs and in other immortalized cell lines. In a time when it was quite a technical challenge to just maintain human ESCs in culture, such low efficiencies were nearly prohibitive for a regular laboratory to perform any precise genome editing in these cells.

Subsequent research on human ESC biology has led to significant advancements that contributed to improved genome editing in human ESCs and iPSCs. The discovery of the pro-survival effects of a specific rho-associated kinase (ROCK) inhibitor is a landmark achievement in stem cell research and helped to overcome the obstacle of poor single-cell survival of hESCs (Watanabe et al. 2007), which was one of the major impediments to advances in many applications related to human pluripotent stem cells. The much improved single-cell survival rates aided by ROCK inhibitors, together with increased cell proliferation rates offered by defined feeder-free culture systems (Chen et al. 2011), facilitate higher gene delivery efficiency by electroporation, and allow selections of rare

clones that have undergone successful genome editing. The most important contributing factor to the recent success of gene corrections in human iPSCs, however, is the development of engineered endonuclease technologies (Bibikova et al. 2001; Christian et al. 2010; Cong et al. 2013; Mali et al. 2013; Porteus and Baltimore 2003).

The fundamental principle behind the new set of genome-editing tools, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPRs), is that DNA double strand breaks (DSBs) at precise genomic locations will activate endogenous cellular DNA repair machinery to facilitate desired genomic modifications (Jasin 1996; Vasquez et al. 2001). DSBs can be repaired by either the error-prone non-homologous end joining (NHEJ) or the high fidelity homology-directed repair (HDR) pathways when a homologous donor template is available. The developments of these designer endonucleases and their underlying mechanisms have been extensively reported and reviewed in the literature and are not discussed here (Collin and Lako 2011; Doudna and Charpentier 2014; Durai et al. 2005; Gaj et al. 2013). Needless to say, these new systems contributed significantly to the research fields of human pluripotent stem cells, which have been more difficult than most other cell types to be precisely genome edited. With the intent of developing gene/cell therapies and advanced disease models, various disease-specific iPSCs have been derived and some of them were subjected to gene correction using the conventional strategies and the new tools. As expected some monogenic diseases, which have been favorite model diseases in the traditional gene therapy research, also attracted more attention from stem cell researchers. The new genome-editing tools facilitate higher efficiencies of gene corrections at endogenous loci and can overcome some major problems, such as insertional mutagenesis and non-physiological expression levels of transgenes, associated with the traditional “gene addition” approaches. Various targeting strategies, most of them taking advantage of the engineered endonucleases, have been developed to achieve functional correction of disease-related genetic abnormalities in patient-specific iPSCs (Table 1).

Corrections of disease-associated genetic mutations in patient-specific iPSCs

Retinal degenerative diseases

Genetic defects can cause several forms of retinal degenerative diseases. One common consequence of the genetic predisposition is the dysfunction or loss of retinal pigment epithelium (RPE), which can further lead to the degeneration of photoreceptors and loss of vision. Patients suffering from retinal degenerative diseases have been anticipated to be among the first to benefit from human pluripotent stem cell-based therapies. One of the features that have made retinal diseases popular candidates for ESC- or iPSC-based cell replacement therapy is the phenomenon of ocular immune privilege (Zhou and Caspi 2010), which makes it more feasible to overcome immune rejection of in vitro generated cell types, such as RPEs, after transplantation. In addition, the transplanted site can be easily monitored with noninvasive techniques. Intensive research has been conducted to derive functional retinal pigment epithelium cells from ESCs and iPSCs (Buchholz et al. 2009; Idelson et al. 2009; Meyer et al. 2009). The protective effects of human iPSC-derived RPE were

demonstrated in pre-clinical animal experiments (Carr et al. 2009). Human ESC-derived RPE cells have already entered clinical trials for the treatment of dry atrophic age-related macular degeneration and Stargardt's macular dystrophy in the United States (Schwartz et al. 2012, 2015). The positive mid-term and long-term outcomes of the trials, including that of safety and visual acuity, suggest that human pluripotent stem cell-derived cells could provide a potentially safe new source of cells for the treatments of various medical disorders requiring tissue repair or replacement (Schwartz et al. 2015). The discovery of human iPSCs provided an additional and potentially more advantageous cell source for transplantation, because the use of iPSCs can further eliminate the concerns of immune rejection. In fact, the RIKEN Center for Developmental Biology in Japan has announced the treatment of the very first patient of macular degeneration with retinal tissues derived from the patient's own iPSCs (Reardon and Cyranoski 2014). However, in the inherited forms of retinal degeneration, the patients' own cells still carry the same disease-associated mutation(s). Gene correction in the patient-specific iPSCs, therefore, is essential before the cells can be used for cell therapy.

One of the first reported in situ gene correction studies in human iPSCs was in fact conducted in cells derived from a patient suffering gyrate atrophy (GA), a form of retinal degenerative diseases, and has a homozygous mutation in the ornithine-δ-aminotransferase (OAT) gene. In this proof-of-principle study, no efficiency-enhancing endonuclease was used. Instead, the investigators constructed a bacterial artificial chromosome (BAC)-based donor to achieve HR and gene correction in one of the mutant alleles (Howden et al. 2011). Several years later, these investigators reported gene correction in iPSCs from another type of retinal degenerative disease retinitis pigmentosa, which is the most frequent inherited form of blindness. This time CRISPR/ Cas9 system was used together with a single-stranded oligonucleotide (ssODN) donor to repair a point mutation in the pre-mRNA processing factor 8 (PRPF8) gene, which causes retinitis pigmentosa (Howden et al. 2015). In the later study, the gene correction was technically not performed in established iPSCs, but was conducted simultaneously with the reprogramming process. The genome-editing reagents and the reprogramming factors were co-delivered into patient fibroblasts to achieve this simultaneous reprogramming and gene correction (Howden et al. 2015). This strategy of combining CRSIPR/Cas9 and ssODN repair donor was also used in a separate genome-editing study, where a point mutation in retinitis pigmentosa GTPase regulator (RPGR) gene was successfully corrected in iPSCs derived from an X-linked retinitis pigmentosa patient (Bassuk et al. 2016).

Alpha-1 antitrypsin deficiency

Alpha-1 antitrypsin deficiency is a common genetic disease caused by the defective production of an enzyme alpha-1 antitrypsin (AAT) in the liver (Perlmutter 2000a). In healthy individuals, the AAT proteins travel from the liver through the blood to protect the lungs and other organs. However, in alpha-1 antitrypsin deficiency patients, they are stuck in hepatocytes due to point mutations, most commonly the E342K Z-AAT mutation, that lead to the misfolding of the protein and the formation of insoluble polymers. The accumulation of excessive mutant Z-AAT protein in hepatocytes can lead to various liver diseases. The lack of normal AAT enzyme in circulation, on the other hand, is the main cause of lung

problems. Currently, there is no effective treatment to remove or prevent the accumulation of mutant Z-AAT protein in the liver (Perlmutter 2000b). Gene therapy for AAT deficiency has been explored by many due to the monogenic nature of the disease (Flotte and Mueller 2011). The transgene expression approach can potentially prevent AAT deficiency lung disease, while the shRNA knock-down approach has the potential to reduce mutant protein accumulation in hepatocytes and treat liver diseases. In comparison to these conventional gene therapy approaches, precise repair of the mutation at endogenous AAT locus has a great advantage and could potentially prevent or cure both liver and lung diseases associated with AAT mutations.

The AAT mutation is one of the first molecular targets in reported studies that applied the modern genome-editing tools in enhancing gene correction efficiency in patient-specific iPSCs. In the first of such studies, Yusa and colleagues designed ZFN pairs to facilitate the correction of the Z-mutation and have demonstrated a low off-target effect of the procedure through the whole exome sequencing (Yusa et al. 2011a). Together with a ZFN-mediated gene correction of a Parkinson's disease-related α-synuclein gene (Soldner et al. 2011), this study demonstrated for the first time the feasibility and efficiency of correcting a disease-causing mutation in iPSCs using an engineered endonuclease (Yusa et al. 2011a).

A greater impact of this study, however, is the introduction of an elegant targeting approach to achieve footprint-free gene targeting. Most of the gene-targeting methods rely on positive selection to isolate rare clones that have undergone homologous recombination. To remove the unwanted selection cassettes after confirming targeted integration, recombination systems, such as Cre/loxP, are commonly used, which would leave behind ~34 bp of loxP sequence in the genome of the gene-corrected cells. To eliminate the potential interference of these small ectopic sequences on transcriptional or epigenetic regulations, Yusa and colleagues developed a novel system that converts the drug selection cassette into piggyBac DNA transposons. The previous studies have shown that the optimized piggyBac transposons can transpose efficiently in human cells and, importantly, can be excised out seamlessly by the transient expression of piggyBac transposase (Yusa et al. 2011b). By flanking the selection cassette with piggyBac inverted terminal repeat (ITR) sequences, these investigators first examined the feasibility of this strategy in mouse iPSCs before successfully and seamlessly corrected the Z-AAT mutation in human iPSCs (Yusa et al. 2011a). A later study used this piggyBac transposon-based donor and TALENs to correct the Z-AAT mutation in patient-iPSCs and demonstrated a similar efficiency to the ZFN-mediated targeting (Choi et al. 2013). A Cas9-sgRNA combination designed to target the Z-AAT mutation has been shown to be allele-specific; it targets the mutant allele effectively while having negligible effect on the healthy allele that differs by a single nucleotide at the mutation site (Smith et al. 2015).

Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is one of the most common and severe inherited muscle degenerative diseases, and is almost exclusively the results of genetic defects in dystrophin. Dystrophin is a key structural protein of the dystrophin glycoprotein complex (DGC) that bridges the inner cytoskeleton (F-actin) and the extra-cellular matrix. (Muntoni

et al. 2003). Because it is also one of the most difficult genetic diseases to treat by the conventional methods, gene therapy approaches have been intensively pursued. Compared to many other monogenic genetic diseases, developing an effective gene and cell therapy strategy for DMD has been more challenging due to the complexity of dystrophin; it is the largest known gene (~2.4 Mb) with many (79) exons, several promoters, and RNA splicing forms. Disease-causing mutations have been reported in many different forms that ultimately lead to the disruption of protein reading frame and the absence of functional protein (Pichavant et al. 2011). Towards developing stem cell-based gene and cell therapy, human artificial chromosome (HAC) has been used to provide wild-type dystrophin expression in DMD patient-specific iPSCs. By delivering a complete genomic dystrophin sequence into iPSCs, studies have shown the restoration of dystrophin in iPSC-derived skeletal muscles and cardiomyocytes (Kazuki et al. 2010; Zatti et al. 2014).

The development of designer endonucleases facilitated the implementation of novel strategies correcting endogenous dystrophin gene in mouse models and in patient-derived cells (Iyombe-Engembe et al. 2016; Nelson et al. 2016; Ousterout et al. 2013, 2015a, b). These encouraging results led to the efforts in correcting patient-specific iPSCs (Li et al. 2015; Young et al. 2016). Patient-specific iPSC lines with a large genomic deletion, including the deletion of the entire dystrophin exon 44 that resulted in a premature stop codon in exon 45, were used in a study, where the investigators attempted various genome-editing strategies to restore the protein function (Li et al. 2015). Aided by TALENs and Cas9-sgRNAs, Li and colleagues performed the genetic correction of the dystrophin gene using three different methods: (1) disruption of the splicing acceptor to skip exon 45, (2) introduction of small indels to modulate the protein reading frame, and (3) knock-in of the missing exon 44 to restore the full protein coding region. Corrected iPSC clones with a minimal mutation load were identified by karyotyping, copy number variation array, and exome sequencing. Expression of full-length dystrophin protein was detected in skeletal muscle cells differentiated from gene-corrected iPSCs, among which the iPSCs corrected by exon 44 knock-in demonstrated the highest expression level (Li et al. 2015).

In a separate study, Young and colleagues also conducted gene editing in DMD patient-specific iPSCs that carry various mutations in the genomic region around dystrophin exon 44–55, a hotspot for DMD-related mutations. Here, they focused on a strategy that involves excision of exons 45–55 to reframe dystrophin and the creation of an internally deleted protein that is stable and partially functional (Young et al. 2016). The advantage of this strategy is that it can apply to ~60 % of DMD patient that have mutations in this genomic region, regardless of the exact nature of each individual mutation. The effectiveness of this “multi-exon skipping” gene correction method has been shown previously including in studies where patient myoblasts were edited by multiplexed CRISPR/Cas9 (Béroud et al. 2007; Iyombe-Engembe et al. 2016; Ousterout et al. 2015a). Using a similar CRISPR system, Young and colleagues achieved up to 725 kb deletion of the mutated dystrophin gene by NHEJ in DMD patient-specific iPSC lines. Importantly, the functionality of the internally deleted protein was demonstrated upon in vitro differentiation of the targeted iPSCs and in vivo engraftment of the differentiated cells. Skeletal muscle myotubes and cardiomyocytes derived from iPSCs express stable dystrophin that improves membrane

stability. Restoration of dystrophin was also demonstrated after skeletal muscle cells derived from the reframed iPSCs were engrafted into a mouse model of DMD (Young et al. 2016).

Sickle cell disease

Sickle cell disease (SCD) is one of the most common monogenic diseases and one of the first diseases, whose molecular bases were elucidated. A single nucleotide SCD mutation in β-hemoglobin (HBB) gene, which results in a glutamine-to-valine substitution at the sixth residue of the β-globin protein, is responsible for the disease. The simplicity of the molecular basis also prompted significant efforts in developing gene and cell therapy for SCD. Precise repair of the SCD mutation in patient-specific iPSCs, together with efficient differentiation methods, could potentially lead to autologous transfusion or transplantation therapies. The first such study was performed using ZFNs that target DNA sequences near the SCD mutation (Zou et al. 2011a). Due to the high sequence homology among members of the globin gene family (HBB, HBD, HBE, and HBG), Zou, Mali, and colleagues first examined the efficiency and confirmed the HBB specificity of the designed ZFNs using an EGFP reporter system (Durai et al. 2005). After selecting iPSC clones with targeted integration and subsequent removing of drug selection cassette, the authors demonstrated the expression of normal HBB at mRNA level in erythroid cells differentiated from iPSCs. Because HBB gene is transcriptionally inactive in undifferentiated iPSCs, gene targeting at this locus has been extremely difficult and, as shown in this study, can benefit greatly from the development of the new genome-editing tools (Zou et al. 2011a). Several other studies have subsequently shown the efficiency-enhancing effects of ZFNs, TALENs, and CRISPR in targeting and correcting SCD mutation in iPSCs (Huang et al. 2015; Sebastiano et al. 2011; Sun and Zhao 2014).

β-Thalassemia

Unlike in sickle cell disease where a single point mutation is the underlying genetic cause in all patients, thalassemias have several subtypes and a much diverse mutation spectrum. Even in β-thalassemia major (also called Cooley's anemia), a severe form of thalassemia caused by loss of β-globin, and many different mutations in HBB gene have been reported. Currently, there are several published studies that have corrected β-globin mutations commonly found in southern China, an area that has high population frequency of thalassemia (Ma et al. 2013, 2015; Song et al. 2015; Wang et al. 2012; Xie et al. 2014). Based on the facts that various forms of mutations are involved in the disease and that the HBB gene is a relatively small gene, efforts have been made to develop a universal correction strategy to replace the entire coding region of a mutant HBB gene with a healthy copy (Ma et al. 2013; Song et al. 2015). It is an attractive strategy; however, it remains to be determined how robust this strategy will be in a larger number of patient-iPSCs with various forms of HBB mutations.

Severe combined immunodeficiency

Severe combined immunodeficiency (SCID) is another group of genetic diseases that gene and cell therapies have been heavily pursued in the past decades. These are life threatening diseases that allogeneic hematopoietic stem cell transplantation is currently the only established therapy. Similar to sickle cell disease and thalassemias, SCID patients can

benefit from genetically corrected iPSCs that potentially may provide alternative sources for transplantation. Recently, three studies have been reported that iPSCs derived from different forms of SCID have been gene-corrected with TALEN and CRISPR endonucleases (Chang et al. 2015; Howden et al. 2015; Menon et al. 2015). Menon and colleagues used the TALEN technology to correct a splice-site mutation in the interleukin-2 receptor gamma chain (IL-2Rγ) gene, which is required for the differentiation and maturation of the majority of lymphocytes. Loss of function mutations in this gene often results in X-linked SCID. While the patient-derived mutant iPSCs have the defect in generating mature NK cells and T cell precursors, the gene-corrected iPSCs gained the capacity to generate the full spectrum of hematopoietic cells, including lymphocytes that express the correctly spliced IL-2Rγ (Menon et al. 2015). In a separate study, Chang and colleagues successfully correct a JAK3 mutation in SCID-iPSCs using CRISPR technology. The corrected cells also regained the differentiation potentials to T cell progenitors (Chang et al. 2015). Although functional outcomes were not examined in a third study with iPSCs derived from an ADA-SCID patient, Howden and colleagues combined the reprogramming and genome-editing processes together and successfully generated ADA gene-corrected patient-iPSCs in a shortened time frame (Howden et al. 2015). These recent studies highlight the potential for developing novel autologous cell therapies for SCID patients.

Cystic fibrosis

Cystic fibrosis (CF) is a condition that thick sticky mucus forms in the lungs, impairs breathing, and often leads to premature respiratory failure. The primary genetic defects in CF are found in the CF transmembrane conductance regulator (CFTR) gene, which encodes a chloride channel protein that regulates lung epithelial chloride transport. Mutations in CFTR gene prevent the expression or reduce the functionality of this chloride transporter at the cell membrane. To generate human-based disease models for an improved understanding of disease mechanism and to explore the potential of generating autologous lung progenitors for treating CF, two groups have recently conducted gene correction in CF-specific iPSCs (Crane et al. 2015; Firth et al. 2015). Firth and colleagues used CRISPR technology and a piggyBac-based donor to achieve footprint-free gene correction at the CFTR locus in patient-specific iPSCs. They subsequently differentiated the iPSCs into lung epithelial cells and demonstrated recovered function and expression of CFTR (Firth et al. 2015). Crane and colleagues used ZFN technology to achieve gene correction in CFTR gene. Similar to the other study, when the corrected CF-iPSCs were induced into lung epithelial cells, mature CFTR glycoprotein was expressed, which in turn restored CFTR chloride channel function (Crane et al. 2015). In an earlier and related study, Schwank and colleagues have used the CRISPR system to repair CFTR in intestinal stem cell organoids of cystic fibrosis patients and have demonstrated high specificity of CRISPR/Cas9 in the study (Schwank et al. 2013).

Trinucleotide repeat expansion disorders

Trinucleotide repeat expansion disorders are a group of genetic diseases caused by trinucleotide repeat expansion. The trinucleotide repeat expansion, an increase in the repeat count, can result in defect of a gene, where the repeat expansion occurs. This plays a role in several forms of neurodegenerative diseases, such as Huntington’s disease (HD) and fragile X syndrome (Pearson et al. 2005). This unique genetic feature of these disorders represents a

different challenge in gene correction than other genetic diseases. An and colleagues first addressed this challenge using Huntington's disease-specific iPSCs, where the CAG repeat expansion in huntingtin (HTT) gene is the underlying genetic cause of HD (An et al. 2012). They initially used a traditional HR approach to delete the expanded CAG repeats without designer nucleases and in a later study demonstrated that CRISPR/Cas9 can further enhance the HR efficiency in their system (An et al. 2014). In parallel to the HD-iPSC studies, Park and colleagues also used CRSIPR but with a different approach to delete the disease-associated CGG repeats in the 5′-UTR of the fragile X mental retardation 1(FMR1) gene in iPSCs derived from fragile X syndrome patients. Instead of using an exogenous homology donor template, the investigators took advantage of the high efficiency of Cas9 in generating DSBs and triggering NHEJ to delete the abnormal CGG repeat expansion (Park et al. 2015a). In both the HD and fragile X syndrome gene correction studies, the deletion of trinucleotide repeat expansions in patient-iPSCs resulted in restoration of gene expression and reversal of susceptibility to cell death in neural stem cells derived from corrected iPSCs, suggesting that the genome-editing approach could provide alternative therapies to these neurodegenerative diseases.

Other diseases

The above-mentioned gene corrections were performed in iPSCs derived from patients of diseases that have been extensively studied for gene or cell therapy development over the past decades. It comes as no surprise that they also attracted more attention from stem cell investigators. In addition to these diseases, recent studies also demonstrated successful in situ gene corrections in iPSCs derived from pyruvate kinase deficiency (Garate et al. 2015), familial platelet disorder (Connelly et al. 2014; Iizuka et al. 2015), hemophilia A (Park et al. 2015b), Hutchinson–Gilford progeria syndrome (Liu et al. 2011), tauopathies (Fong et al. 2013), ALS (Kiskinis et al. 2014), and Niemann–Pick type C disease (Maetzel et al. 2014) (Table 1). Although in most of these cases, successful corrections of disease-associated mutations only represent a very early stage in developing stem-based therapy, the resultant cell lines will no doubt provide valuable tools for disease modeling and drug screening purposes.

Diseases that require genetic corrections before reprogramming

Patient-specific iPSCs have been generated from many diseases, but it is rather challenging to reprogram somatic cells from certain patients, such as those suffer from Fanconi anemia (FA). FA is characterized by chromosomal instability and progressive bone marrow failure, as results of defects in the FA DNA repair pathway. These mutations not only have detrimental effects on maintaining genomic integrity, but also significantly decrease the reprogramming efficiencies (Yung et al. 2013). One strategy that has been successful in generating FA iPSCs is by FA pathway complementation, such as using lentiviral vectors expressing FANCA or FANCD2, before or during the reprogramming process (Müller et al. 2012; Raya et al. 2009). An additional advantage of this "correction first" approach is that the generated iPSCs would be able to maintain a higher level genomic integrity compared to those that are defective in the DNA repair pathways. Although viral vectors were used in these earlier studies to correct the genetic pathways before reprogramming, the recent study by Howden and colleagues has demonstrated the feasibility of simultaneous reprogramming

and precise genome editing by CRISPR (Howden et al. 2015). This approach may prove to be valuable in generating and correcting iPSCs from patients, whose genetic defects are detrimental to the reprogramming process.

The safe harbor gene correction approach

For a disease that a loss-of-function gene mutation is the primary cause, a potent alternative approach to in situ gene correction is the targeted transgene integration at a safe harbor locus, such as the AAVS1 site (Hockemeyer et al. 2009; Smith et al. 2008). The safe harbors provide favorable epigenetic features that facilitate a more consistent expression of transgenes across different cell types (Ogata et al. 2003; Smith et al. 2008). Disruption of AAVS1 locus in human genome did not seem to have detrimental consequences to cellular function. Furthermore, effective tools based on various endonuclease systems for targeting AAVS1 site have been developed and can be used for delivering various targets (Hockemeyer et al. 2009, 2011; Mali et al. 2013). Therefore, this is an attractive approach that can overcome the transgene silencing and insertional mutagenesis problems associated with the traditional virus- or transposon-based gene-addition approaches. This approach has been shown to rescue disease phenotypes in iPSCs derived from patients of chronic granulomatous disease (CGD) and Glanzmann thrombasthenia (Dreyer et al. 2015; Merling et al. 2015; Sullivan et al. 2014; Zou et al. 2011b). Since each gene-specific endonuclease has a unique set of potential off-targets, using the validated and universal genome-editing tools targeting AAVS1 site offers an additional advantage in the safety evaluation of the targeted cell lines.

Current challenges and future perspectives of iPSC-based gene therapy and disease modeling

Maintaining genome integrity in gene-corrected iPSCs

Because the new genome-editing tools enhance efficiency by creating DNA strand breaks at pre-determined genomic loci, an obvious concern is the potential off-target effects of the engineered endonucleases, which have been reported by several studies (Fu et al. 2013; Gabriel et al. 2011; Hsu et al. 2013; Pattanayak et al. 2011, 2013). The reported experiments in these studies were mainly conducted in transformed or cancer cell lines commonly used in research laboratories. A key question is then whether the reported substantial off-target effects would be observed in primary cells or human stem cell lines, such as iPSCs, which rely more heavily than cancer cell lines on the efficiency-enhancing nucleases for achieving sufficient genome editing. By the whole genome sequencing of gene-targeted human iPSC lines, several groups have demonstrated that the off-target effects of both TALENs and CRISPR/Cas9 appear to be minimal (Smith et al. 2014; Suzuki et al. 2014; Veres et al. 2014). The majority of gene correction studies reviewed here also reported negligible off-target mutations by ZFNs, TALENs, and CRISPR in patient-specific iPSCs (Table 1), suggesting that the fidelity of the current genome-editing technologies in human stem cells is significantly higher than that observed in other transformed cell lines. However, it is worth to note that although the current high throughput sequencing technologies (e.g., whole genome sequencing) and bioinformatics are powerful in detecting single nucleotide

variations (SNVs), they are not yet optimal in detecting small indels, which are the types of mutations that most likely to occur as results of off-target cleavage by nucleases. This issue will likely be addressed in the near future because of the rapid advancement in sequencing technology and computational biology. In the meantime, various strategies have been developed to further enhance the specificity of CRISPR, currently the most popular genome-editing tool. These strategies include decreasing the length of the gRNA–DNA interface (Fu et al. 2014), using the nickase form of Cas9 (Ran et al. 2013; Shen et al. 2014) or catalytically inactive Cas9 fused to FokI catalytic domain (Tsai et al. 2014). More recently, progress has also been made to further improve Cas9 nuclease specificity while retaining the on-target activities, through structure-guided protein engineering (Kleinstiver et al. 2016; Slaymaker et al. 2016).

To develop better disease models or clinical products, precise genome editing without accessary DNA sequences, such as the loxP site sequence, in the genome is preferred. The utilization of excisable piggyBac transposon in homology donor design represents a significant improvement, because footprint-free genome editing is feasible with this technique (Yusa et al. 2011a). However, this is still a two-step procedure with each step requiring significant time for single clone expansion. This is a potential issue in maintaining genome integrity in iPSCs. Although the fidelity of endonucleases will continue to improve, the numbers of studies have found that genomic alterations can occur in gene-targeted iPSCs due to the prolonged cell culture and/or clonal selection (Firth et al. 2015; Garate et al. 2015; Howden et al. 2011; Ma et al. 2015; Smith et al. 2014). Reducing the total amount of time required for generating gene-edited iPSCs will likely result in reduced genetic abnormality. One strategy to reduce the time required for obtaining the final cellular products is to use ssODNs or other types of homology donors without selection cassette (Soldner et al. 2011; Yang et al. 2013). However, the low HR rates even in the presence of a designer nuclease prevent the widespread application of this approach. Research on the balance between NHEJ and HR will likely help to address this issue and to develop strategies that can tip the balance and promote homology-directed repair. Encouraging results have been reported along this line of research. Several small molecular compounds were identified to affect the ratio of different types of DNA repair (Chu et al. 2015; Maruyama et al. 2015; Pinder et al. 2015; Yu et al. 2015). A strategy that combines cell cycle synchronization techniques with direct delivery of pre-assembled Cas9 ribonucleoprotein (RNP) complexes has been shown to improve HDR in human cells, including ESCs (Lin et al. 2014). A recent study on the kinetics of CRISPR-DNA strand interactions also demonstrated that a rational design of ssODNs can further increase the rate of HDR in human cells (Richardson et al. 2016).

With the rapid advancement in the whole genome sequencing technologies, it should become a standard practice in the near future to sequence the final gene-corrected clones and select the ones with the least mutation load. This is an advantage of iPSCs. However, to generate sufficient numbers of functional cell types for therapies, the iPSCs will need to go through many rounds of differentiation and replication. Considering there are on average greater than three somatic mutations occur per genome per mitotic division (Kinde et al. 2011; Lynch 2010), the eventual therapeutic cell populations will inevitably carry a large number of mutations unrelated to the genome-editing processes. Whether this will pose a potential safety concern for iPSC-based gene/cell therapy remains to be analyzed.

Generation of relevant and functional cell types from iPSCs

The development of ZFN, TALEN, and CRISPR technologies has greatly enhanced our capability to conduct precise genome editing, including the correction of disease-associated mutations. Although the technologies are still far from ideal, in some cases, they are probably sufficient for generating genetically modified cells that are safe and effective for clinical applications (Tebas et al. 2014). In comparison, our limited ability to generate iPSC-derived cell types with sufficient functionality and safety for cell therapy is perhaps a more significant hurdle in clinical translation of the technologies. A review of the literature will show that a significant portion of the published gene-editing studies in patient-iPSCs was conducted in sickle cell anemia, thalassemia, SCID, and other hematologic diseases (Table 1), the ones that theoretically can be treated with transplantation of gene-corrected hematopoietic stem cells (HSCs), T cell progenitors, or transfusion of in vitro generated red blood cells (RBCs). However, no clinical translation of these studies is currently in sight despite the fact that bone marrow transplantation and blood transfusion are both routine medical procedures. The missing link is the generation of iPSC-derived transplantable HSCs with lymphoid and myeloid potentials (Kaufman 2009; Vo and Daley 2015). In vitro technologies to generate large quantity of mature RBCs with adult globin expression are also lacking, even though proof-of-principle experiments have been conducted (Huang et al. 2015). This challenge is by no means specific to blood cell regeneration; uncertainties remain in many diseases related to other organs or tissues. For example, in the case of liver and lung regeneration, it is not yet clear what cell types derived from human iPSCs would be the most suitable for cell replacement therapy. Would the primitive stem cells, progenitor cells or mature-like cell types be more effective? The answer may not be a simple one and it is possible that various cell types at different developmental stages will be needed depending on the nature of the disease and the degree of tissue degeneration. A deeper understanding of developmental biology and additional research in bioengineering and pre-clinical animal models is critically needed to address these important issues.

Conclusions

The past 5 years have seen an explosion of success in precise genome editing of human iPSCs. Taking advantage of the advanced genome-editing tools, investigators have developed innovative strategies to correct many forms of genetic defects in patient-specific iPSC. These studies have demonstrated the effectiveness of the ZFN, TALEN, and CRISPR technologies as well as the high fidelity of these technologies in human iPSCs. The gene-corrected iPSCs will provide a basis for establishing novel disease models and for developing pre-clinical models. Some remaining concerns over the efficiency and fidelity of the technology will like be addressed in the near future by the rapidly advancing genome editing and sequencing technologies. Significant efforts will still be needed to differentiate iPSCs into functional cell types that are essential in translating these valuable cellular resources into effective research and therapeutic tools.

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Table 1

In situ gene corrections in patient-specific iPSCs

Disease origin	Gene	Mutation(s) corrected	Genome-editing tools	Outcome of gene correction	Genome integrity of corrected iPSCs	Study
Retinal degenerative disease/gyrate atrophy	ornithine-δ-aminotransferase (OAT)	C677T (A226V) point mutation	BAC-based HR donor[a]	One copy corrected	High-density aCGH and exome sequencing showed no increase in mutational load in gene-corrected iPSCs	Howden et al. (2011)
Retinal degenerative disease/retinitis pigmentosa	precursor mRNA-processing factor 8 (PRPF8)	C > T point mutation in exon 42	CRISPR/Cas9 with ssODN donor[b]	Correction confirmed by sequencing	Normal karyotypes. Free of residual plasmid sequences	Howden et al. (2015)
Retinal degenerative disease/X-linked retinitis pigmentosa (XLRP)	retinitis pigmentosa GTPase regulator (RPGR)	G3070T point mutation	CRISPR/Cas9 with ssODN donor	Deep sequencing of transfected cells showed that 13 % of reads contained precise correction of the mutation	N.A.	Bassuk et al. (2016)
Alpha-1 antitrypsin deficiency	Alpha-1 antitrypsin (AAT)	Z-AAT point mutation (E342K)	ZFNs and piggyBac donor	Wild-type AAT expression restored	Exome sequencing demonstrated minimal mutational effect of gene correction	Yusa et al. (2011a)
		Z-AAT point mutation (E342K)	TALENs and CRISPR/ Cas9 with piggyBac donor	Reduction of AAT accumulation in hepatocytes derived from corrected iPSCs	Targeted deep sequencing detected no off-target cleavage by CRISPR in iPSCs	Choi et al. (2013), Smith et al. (2015)
Sickle cell disease	β-Hemoglobin (HBB)	SCD point mutation	ZFNs	Wild-type HBB transcript detected	Genome-wide SNP concordance analysis did not detect overt difference after gene correction	Zou et al. (2011a)
			ZFNs	HBB gene corrected	No ZFN-induced mutation in paralogous globin genes or other predicted off-targets	Sebastiano et al. (2011)

Disease origin	Gene	Mutation(s) corrected	Genome-editing tools	Outcome of gene correction	Genome integrity of corrected iPSCs	Study
			TALENs with piggyBac transposon-based donor	HBB gene corrected	Surveyor assay detected no mutations in the top six predicted off-targets	Sun and Zhao (2014)
			CRISPR/Cas9	β-Globin protein expression detected in mature erythrocytes differentiated from genome-edited iPSCs	Deep sequencing did not detect alterations at predicted off-target sites of Cas9-sgRNA	Huang et al. (2015)
β-Thalassemia	β-Hemoglobin (HBB)	Homozygous 41/42 deletion	Homology donor-mediated HR[a]	Expression of human β-globin detected in recipient mice	Normal karyotype	Wang et al. (2012)
		Homozygous IVS2–654 (C → T point mutation); β41–42 (−TCTT)	TALENs and ZFNs	HBB mRNA restored in in vitro differentiated cells	Numbers of CNVs, indels and SNVs identified in resultant iPSCs, unlikely caused by ZFNs	Ma et al. (2013, 2015)
		−28 (A/G) mutation of the promoter and the 4 bp (TCTT) deletion at codon 41 and 42 of exon 2	CRISPR/Cas9 with piggyBac-based donor	Expression of wild-type HBB in erythroblast differentiation from corrected iPSCs	Sanger sequencing detected no mutation in 6 predicted off-targets	Xie et al. (2014)
		β17/17 Homozygous point mutation	CRISPR/Cas9	Restored HBB expression and reduced reactive oxygen species production	Normal karyotype	Song et al. (2015)
Severe combined immunodeficiency (SCID)	Interleukin-2 receptor gamma chain (IL-2Rγ), X-linked	A splice-site mutation, c.468 + 3A > C	TALENs	In vitro generation of mature NK cells and T cell precursors expressing the correctly spliced IL-2Rγ	Whole exome sequencing detect no off-target mutations	Menon et al. (2015)
	JAK3	Homozygous C > T point mutation (p.R613X) in exon 14	CRISPR/Cas9	Differentiation potential of early T cell progenitors from iPSCs restored	WGS of three corrected iPSC lines demonstrated that no mutations (SNVs nor indels) were introduced into the 1450	Chang et al. (2015)

Disease origin	Gene	Mutation(s) corrected	Genome-editing tools	Outcome of gene correction	Genome integrity of corrected iPSCs	Study
					potential off-target sites	
	Adenosine deaminase (ADA)	C > T transition (1081C > T) in exon 11	CRISPR/Cas9 with ssODN donor[b]	Correction confirmed by sequencing	Normal karyotypes. Free of residual plasmid sequences	Howden et al. (2015)
Pyruvate kinase deficiency (PKD)	PKLR	(1) Heterozygous 359C > T in exon 3 and 1168G > A in exon; (2) homozygous IVS9 (+1) G > C	TALENs and donor vector containing partial cDNA covering exon 3–11	Insertion of partial cDNA at endogenous PKLR locus restored generation of metabolically corrected erythroid cells	CGH and Exome sequencing identified small number of CNVs and SNVs that are likely inherited from rare somatic cells or accumulated during reprogramming and iPSC expansion	Garate et al. (2015)
familial platelet disorder	RUNX1	Y260X nonsense mutation	ZFNs	Restored megakaryopoiesis in FPD iPSC-derived hematopoietic cells	N.A.	Connelly et al. (2014)
		R174X nonsense mutation	TALENs	Restored megakaryopoiesis in FPD iPSC-derived hematopoietic cells	N.A.	Iizuka et al. (2015)
Hemophilia A	Coagulation factor VIII (F8)	Chromosomal inversions that involve introns 1 and 22	CRISPR/Cas9	Endothelial cells from corrected iPSCs expressed the F8 gene and functionally rescued factor VIII deficiency in mouse model of hemophilia	WGS and targeted deep sequencing demonstrated no detectable off-target mutations	Park et al. (2015b)
Duchenne muscular dystrophy	Dystrophin	Large genomic deletion including exon 44	TALENs and CRISPR/ Cas9	Dystrophin protein was detected by myogenic differentiation in the corrected iPSCs	Genomic integrity tests identified minimum off-target mutagenesis by the nucleases	Li et al. (2015)
		Genomic deletion including Exon 46–47; Exon 50 duplication	CRISPR/Cas9	Truncated but in-frame and functional dystro	No off-target activity detected at predicted sites	Young et al. (2016)

Disease origin	Gene	Mutation(s) corrected	Genome-editing tools	Outcome of gene correction	Genome integrity of corrected iPSCs	Study
				phin detected in iPSC-derived muscle cells in vitro and in mouse model of transplantation		
Hutchinson–Gilford progeria syndrome	Lamin A (LMNA)	Multiple	Helper-dependent adenoviral vector (HDAdV)-mediated HR[a]	Restored expression of wild-type lamin A, decelerated senescence and restoration of normal nuclear morphology	Genome-wide SNP array genotyping demonstrated no CNVs generated in gene-corrected iPSCs	Liu et al. (2011)
Parkinson's disease	α-Synuclein (α-SYN)	G209A (A53T) point mutation	ZFNs	RT-PCR confirmed loss of mutant expression	Genome-wide analysis of probable off-targets showed no evidence of off-target genome disruption	Soldner et al. (2011)
Trinucleotide repeat expansion disorders/ Huntington's disease	Huntingtin (HTT)	Expanded CAG repeats	Homology donor-mediated HR with and without CRISPR/Cas9	Normalized HD signaling pathways and reversed susceptibility to cell death in neural stem cells	Normal karyotype	An et al. (2012, 2014)
Trinucleotide repeat expansion disorders/ fragile X syndrome	Fragile X mental retardation 1 (FMR1)	Expanded CGG repeats	CRISPR/Cas9- mediated deletion	Ablation of the repeats restored FMR1 mRNA and protein expression as well as demethylation in FMR1 promoter	N.A.	Park et al. (2015a)
Tauopathies	microtubule-associated protein tau (MAPT)	TAU-A152T point mutation	ZFNs	Generation of healthy neurons with smooth TAU and MAP2 staining, eliminating TAU fragments in neurons	Normal karyotype	Fong et al. (2013)
Amyotrophic lateral sclerosis (ALS)	Superoxide dismutase 1 (SOD1)	SOD1-A4V missense mutation	ZFNs	Rescued survival and size defects in iPSC-derived motor neurons	WGS and Nanostring nCounter Karyotyping Panel analysis demonstrated lack of large-scale CNVs or off-target cleavage	Kiskinis et al. (2014)

Disease origin	Gene	Mutation(s) corrected	Genome-editing tools	Outcome of gene correction	Genome integrity of corrected iPSCs	Study
Niemann–Pick type C (NPC) disease	NPC1	I1061T	TALENs and piggyBac-based donor	Cholesterol and autophagy defects rescued in hepatic and neuronal cells from iPSCs	No mutation observed in top ten predicted off-target sites. No CNVs observed genome-wide	Maetzel et al. (2014)
Cystic fibrosis	cystic fibrosis transmembrane regulator (CFTR)	ΔF508	CRISPR/Cas9 with piggyBac-based donor	Recovery of CFTR function in airway epithelial ells derived from corrected iPSCs	Whole exome sequencing. No mutation in predicted off-target sites. SNVs detected but likely not due to gene correction process	Firth et al. (2015)
		ΔF508 and ΔI507	ZFNs	Restoration of mature CFTR protein and chloride channel function in iPSC-derived epithelial cells	No CNV identified by CGH and WGS. No mutation identified by WGS at the top 100 potential off-targets. Two and eight coding SNVs found in two corrected iPSC lines, unlikely induced by ZFNs	Crane et al. (2015)

N.A. not analyzed or reported

[a] Genome editing conducted without the aid of any engineered endonuclease

[b] Genome-editing reagents co-delivered with reprogramming factors to patient fibroblasts for simultaneous reprogramming and genome editing