From Genomics to Gene Therapy: Induced Pluripotent Stem Cells Meet Genome Editing

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Abstract

The advent of induced pluripotent stem (iPS) cells has opened up numerous avenues of opportunity for cell therapy, including the initiation in September 2014 of the first human clinical trial to treat dry age-related macular degeneration. In parallel, advances in genome-editing technologies by site-specific nucleases have dramatically improved our ability to edit endogenous genomic sequences at targeted sites of interest. In fact, clinical trials have already begun to implement this technology to control HIV infection. Genome editing in iPS cells is a powerful tool and enables researchers to investigate the intricacies of the human genome in a dish. In the near future, the groundwork laid by such an approach may expand the possibilities of gene therapy for treating congenital disorders. In this review, we summarize the exciting progress being made in the utilization of genomic editing technologies in pluripotent stem cells and discuss remaining challenges toward gene therapy applications.

INTRODUCTION

Pluripotent Stem Cells

In complex organisms, a single fertilized egg has the capacity to develop into a multicellular entity with distinct organs that perform specialized functions. Before implantation into the uterus early in development during the blastocyst stage, embryonic stem (ES) cells are isolated from the inner cell mass of an embryo. When cultured in vitro, these ES cells retain pluripotency, which is the ability to become any type of specialized cell in an organism. Although it is enigmatic as to why and how ES cells, but not other somatic cells, are able to maintain pluripotency when cultured, attempts by researchers to address these questions from various angles have been hampered by inadequate genetic tools, which are critical for precisely investigating the function of individual genes or genetic networks. However, advances over the past 30 years have provided us with myriad options to elucidate the so-called black box of ES cell pluripotency.

Establishment of Mouse Embryonic Stem Cells

In 1981, mouse pluripotent stem cells were first isolated from mouse blastocysts (129 SvE strain) by Dr. Martin Evans (36), and the cells were named ES cells by Dr. Gail Martin (87). Mouse ES cells grow rapidly on a fibroblast-feeder layer while retaining the ability to develop into a mouse blastocyst and, ultimately, a chimeric mouse. ES cells sometimes differentiate into germ cells, resulting in the generation of an offspring whose entire body consists of ES cell–derived cells. The ability to culture ES cells marked the first step in advancing our knowledge about pluripotency mechanisms.

Gene Targeting Technology in Mice

In the early 1980s, gene targeting by homologous recombination (HR) became possible in cultured mammalian cells (38, 122). Soon after, the technology was applied to mouse ES cells (136) and demonstrated relatively higher HR rates compared with other cell types. As genetically modified ES cells can contribute to chimeric mice and offspring, gene targeting in mouse ES cells has today become a popular approach to generate transgenic or knockout mice (14).

Identification of Pluripotency Regulating Genes

For nearly a decade and a half, several groups have attempted to define the molecular foundation of pluripotency by knocking out pluripotency-related genes in ES cells. We and others have identified dozens of genes that are associated with pluripotency or self-renewing capacity in mouse ES cells, such as *Oct3/4* (96, 97), *Nanog* (17, 92), *Sox2* (2), *ERas* (128), *Klf4* (76), *c-Myc* (15), and *Sox15* (88). From extensive analyses of these genes, we began to realize that a pluripotent state is maintained as a network by a subset of core transcriptional factors (9). Building upon previous knowledge that terminally differentiated somatic cells can be reprogrammed into a totipotent state by somatic nuclear transfer (50, 147) or a pluripotent state by fusion with ES cells (28, 127), these observations suggested that supplementing somatic cells with the right external factors from ES cells may reconstitute an ES-like pluripotent state.

Generation of Induced Pluripotent Stem Cells

In 2006, we reported the identification of four reprogramming factors, *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*, that were sufficient to convert somatic cells to a pluripotent state, which we termed induced pluripotent stem cells, or iPS cells (129, 130). Generating iPS cells from readily available somatic cells, such as skin fibroblasts or peripheral blood cells, eliminated the need for fertilized embryos,

and opened the door for establishing personalized pluripotent stem cells, including those derived from disease patients (103). As long as a differentiation protocol is available, patient-derived iPS cells can be used to obtain any desired cell type for studying cellular disease phenotypes. Control iPS cell lines from healthy individuals can be used; however, one caveat is the genomic variation of donors or individuals. Thus, one should genetically match isogenic control clones (59). In this regard, genetic manipulation of iPS cells, such as correcting disease-related mutations or introducing relevant mutations, would play a critical role in disease modeling and beyond.

GENETIC MANIPULATION OF PLURIPOTENT STEM CELLS

Gene Targeting

Specific gene modification is referred to as gene targeting. By using a host DNA repair pathway, via HR, an endogenous genomic locus can be replaced with an exogenous sequence when supplemented with a targeting vector. Gene targeting enables scientists to have control over the cellular genome through gene deletion or through replacement with a gene of interest, including a foreign gene. Here, we summarize the history of gene targeting, particularly in mouse and human pluripotent stem cells.

Classical Targeting Vector

Initial triumphs in the gene targeting of mouse ES cells were achieved by using a targeting vector, which employs long (5–10 kb) and short (1–4 kb) homology arms on both sides (**Figure 1**). Owing to the low frequency of targeting events in general, the classical targeting vector also contained a drug-selection cassette, such as a neomycin resistance gene derived from a ubiquitous *PGK* gene promoter, for positive selection. If the selection cassette is dispensable after targeting, then it can be flanked by two loxP sequences for excision by a *Cre* recombinase. To eliminate the random integration events, a negative selection marker, such as the thymidine kinase (*tk*) gene from the herpes simplex virus (HSV) (86) or the *diphtheria toxin A* (*DTA*) gene, might be employed outside of the homology arms of the targeting construct.

Gene Targeting in Human Pluripotent Stem Cells

Owing to the fragile nature of human pluripotent stem cells when dissociated into single cells, in addition to their low transfection frequency, gene targeting in human ES cells presented a bigger challenge than mouse ES cells. The first report of HR in human ES cells, in 2003 by Dr. James Thomson's group (162), showed the disruption of the HPRT locus and knockin of a green fluorescent protein (GFP) reporter into the *OCT3/4* (=*POU5F1*) locus. By using a conventional gene targeting vector with 1–20-kb homology arms, however, only a limited amount of success has been reported (139).

An important contribution to improving the handling of human pluripotent stem cells was made by Dr. Yoshiki Sasai's team when it discovered a selective inhibitor of Rho-associated kinase (ROCKi) Y-27632 (145). The inhibitor significantly suppressed the apoptosis of human pluripotent stem cells when dissociated, enabling cells to be electroporated and subcloned more easily.

Conversion of Cell States for Targeting

Human ES cells were isolated from human blastocysts (137); however, their appearance differed from mouse ES cells in cell morphology, growth factor requirements, and epigenetic status. Later,

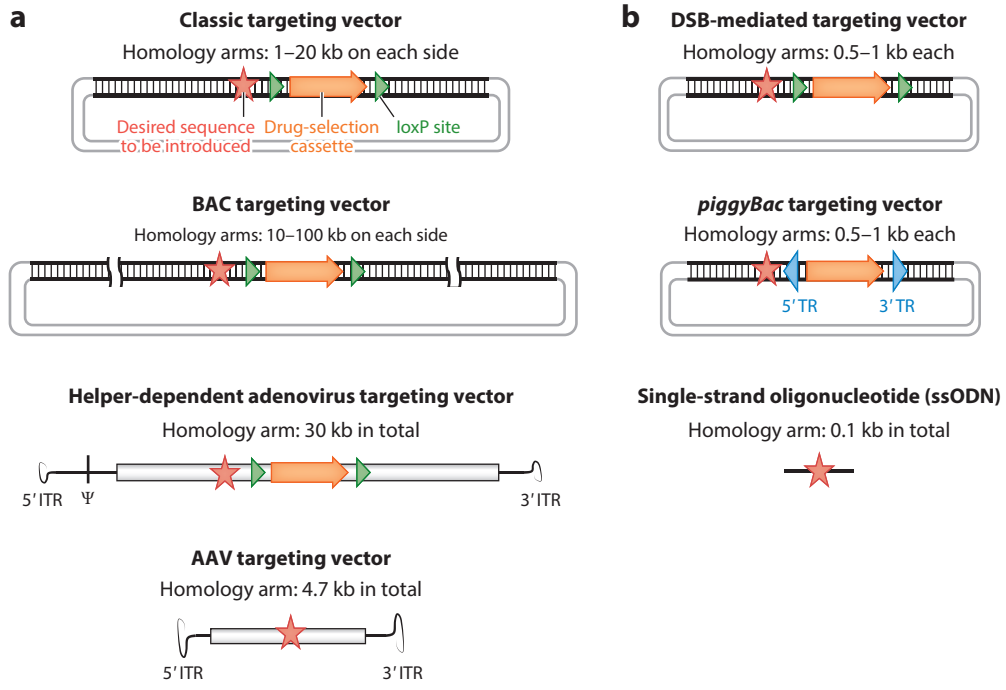

Figure 1

Transition of various targeting vectors. Targeting technologies evolved from (*a*) classic targeting vectors and virus-mediated targeting vectors to (*b*) double-strand break (DSB)-mediated targeting vectors (*right side*) introduced by site-specific nucleases such as TALENs (transcription activator–like effector nucleases) or CRISPR (clustered regularly interspersed short palindromic repeats)-Cas9 systems. The drug-selection cassette is critical to select for rare targeted clones, which may be omitted from some vectors owing to space limitation. Abbreviations: AAV, adeno-associated virus; BAC, bacterial artificial chromosome, ITR, inverted terminal repeat; TR, terminal repeat.

the isolation of mouse EpiSC (10, 134) established that the differences of the mouse embryonic stem (mES) cells and human embryonic stem (hES) cells mainly stem from differences in developmental stages, rather than species-related differences (95). Interestingly, Buecker et al. (11) reported a higher success rate of *HPRT* gene targeting at the naïve state during the reprogramming of human somatic cells by *OCT3/4*, *SOX2*, *KLF4*, *C-MYC*, *NANOG*, and LIF. To date, several groups have reported conversion methods of primed human pluripotent stem cells to a naïve state (43, 131, 135). From a genomics point of view, naïve human pluripotent stem cells are attractive to work with, but molecular mechanisms to distinguish the differences of targeting efficiencies are of great interest and need further exploration.

Bacterial Artificial Chromosome–Mediated Targeting

Length of the homology arms is an important factor for successful gene targeting (50a). To elongate the arm length, BAC (bacterial artificial chromosome) DNA with longer (~100 kb) homology arms has been employed as a donor template (**Figure 1**). In this approach, HR in hES cells has been reported to homozygously disrupt the ATM or p53 genes (124), or to knockin a GFP reporter at the *OSR1* gene locus (81). Because HR events with the BAC donor remain rare, further improvements are required.

Viral Vector–Mediated Targeting

Apart from DNA-based donor vectors, viral vector–mediated targeting approaches have been employed to take advantage of their high transduction efficiency. Adenovirus is a double-stranded DNA virus with an approximate genome size of 30 kb. In place of its viral coding genes, the helper-dependent adenoviral vector (HDAdV) instead carries homology arms and a drug resistance cassette for targeting experiments. Because of the high transduction efficiency and presumably the stability of viral genomic DNA inside of cells, high HR efficiency can be achieved by HDAdV (1, 125). Likewise, adeno-associated virus (AAV), a single-stranded DNA virus with a 4.7-kb genome, is also used as a targeting vector. The packaging size is limited compared with adenovirus, but AAV-mediated targeting can induce relatively high recombination efficiencies in human cells, including pluripotent stem cells (66). However, complications related to the construction and production of HDAdV and AAV are major obstacles that must to be overcome before the virus can be widely used by scientists (75). Biosafety precautions are also required during virus-mediated gene targeting.

THE DEVELOPMENT OF ENGINEERED NUCLEASES

A double-strand break (DSB) in genomic DNA is a critical form of DNA damage that host cells repair immediately to maintain the integrity of the genome and the precious information it encodes. There are several pathways to repair DSBs, such as nonhomologous end joining (NHEJ), HR, and microhomology-mediated end joining. Importantly, by hijacking the host DNA repair pathway with custom-engineered nucleases required to introduce site-specific DSBs, targeting efficiencies via HR can be greatly enhanced.

Meganuclease (Homing Nuclease)

During initial attempts to induce site-specific DSBs, the I-Sce I homing endonuclease (also known as a meganuclease), derived from the mitochondrial DNA of yeast, was used to introduce a site-specific DSB (112, 113). By inserting the 18-bp recognition sequence of I-Sce I into mammalian genomic DNA, a site-specific DSB can be introduced, significantly enhancing HR events by more than two orders of magnitude (24). The HR efficiency was high enough to be performed with homology arms 2.5 kb and 0.3 kb in length. To expand this technique to any desired endogenous sequence, the development of programmable nucleases is required. Owing to limitations of the naturally existing meganucleases, the DNA recognition domain has been engineered to alter their binding specificity (18, 35, 49). However, engineering a meganuclease is a challenge because the DNA recognition domain is tightly associated with the overall protein structure.

Zinc-Finger Nuclease System

The zinc-finger DNA recognition domain is one of the most abundant DNA binding modules in the mammalian genome and has been engineered to bind to a wide range of DNA sequences (33, 60, 100). By conjugating zinc-finger domains with a sequence-independent nuclease domain from the FokI restriction enzyme, zinc-finger nucleases (ZFNs) were developed to specifically generate a DSB only when it is dimerized at the target site (7, 121). ZFNs offer a greater degree of freedom for designing a target sequence than do meganucleases, as the zinc-finger domain can be assembled as modules (141).

In fact, ZFNs have been applied to correct a disease-associated mutation of the endogenous *IL2Rγ* (also known as *IL2RG*) gene for X-linked severe combined immune deficiency (SCID) in the K562 myelogenous leukemia cell line and human primary $CD4^+$ T cells (140). In addition,

Table 1 Examples of zinc-finger nuclease–mediated genome editing in human pluripotent stem cells

Target gene locus	Gene editing	Reference
CCR5	Knockin GFP (green fluorescent protein) expression cassette in hES (human embryonic stem) cells, delivered by IDLVs (integration-defective lentiviral vectors)	78
PIG-A	Knockin to disrupt gene function	159
AAVS1, *OCT3/4*, *PITX3*	Knockin Dox-inducible GFP expression cassette into *AAVS1* locus Knockin GFP for *OCT3/4* or *PITX3* reporter cell lines	52
AAVS1	Knockin shRNA expression cassette in hES cells	30
CCR5	Knockin GFP and Puro-resistance cassette to disrupt *CCR5* gene in hES cells and human iPS (induced pluripotent stem) cells	153
α-Synuclein (SNCA)	Knockin loxP-flanked Puro-resistance cassette to introduce a point mutation in hES cells and human iPS cells	123
AAVS1	Knockin Puro-resistance gene and *CYBB* (=gp91^{phox}) gene to overexpress in human iPS cells	161
β-Globin (*HBB*)	Knockin loxP-flanked Hygro-resistance cassette to correct a point mutation in human iPS cells	160
α1-Antitrypsin (*A1AT*)	Knockin *piggyBac* Puro-TK cassette to correct a mutation in human iPS cells	155
β-Globin (*HBB*)	Knockin loxP-flanked Puro- or Neo-resistance cassette to correct a point mutation in human iPS cells	118
AAVS1	Knockin Puro-resistance cassette and α-globin (*HBA1*) gene in human iPS cells	19
DYRK1A on chromosome 21	Knockin XIST noncoding RNA in human iPS cells	61
LGR5	Knockin GFP reporter in hES cells to isolate intestinal stem cells	40
SOD1	Knockin loxP-flanked Puro-resistance cassette to correct a point mutation (A4V) in human iPS cells	69
TAU (MAPT)	Knockin wild-type sequence to correct a single base mutation (A152T) in human iPS cells	39
AAVS1	Knockin phagocyte oxidase (phox) subunit genes in human iPS cells	90
AAVS1	Knockin GRN (granulin) cDNA in hiPS cells	109

ZFN-mediated gene knockins and knockouts have been investigated in human iPS and ES cells (78). By insertion of a hygromycin resistance cassette, the endogenous *PIG-A* gene was deleted to disrupt the presentation of glycosylphosphatidylinositol (GPI)-anchored proteins on the cell surface in more than 80% of ZFN-treated and drug-selected cells (159). Additionally, the generation of knockin hES/iPS cells for targeting the *OCT3/4*, *AAVS1*, or *PITX3* gene locus has been greatly facilitated by the use of ZFNs (52). **Table 1** provides examples of ZFN-mediated genome-editing technologies applied in human pluripotent stem cells.

Hence, ZFNs represent a powerful tool to modify genomic sequences in cultured human cells. However, the broad use of ZFNs has been compromised because of (*a*) their bias for higher GC content in target sequences (68), (*b*) the absence of certain triplet sequences (3, 119), (*c*) cytotoxicity owing to off-target effects (27), (*d*) context-dependent cleavage activity, and (*e*) patent restrictions.

TALEN System

A novel DNA binding domain known as transcription activator-like effector (TAL effector or TALE) was discovered from *Xanthomonas*, a plant pathogen. The DNA binding domain of TALEs consists of 10 to 30 tandem repeats of RVD (repeat variable di-residue) domains. Each RVD domain recognizes a single base pair by using two amino acid residues (8, 94). Such a

Table 2 Examples of transcription activator–like effector nuclease (TALEN)-mediated genome editing in human pluripotent stem cells

Target gene	Gene editing	Reference
AAVS1, *OCT3/4*, *PITX3*	Knockin Dox-inducible green fluorescent protein (GFP) expression cassette into *AAVS1* locus Knockin GFP for *OCT3/4* or *PITX3* reporter cell lines	53
AKT2, *ANGPTL3*, *APOB*, *ATGL*, *C6OR106*, *CELSR2*, *GLUT4*, *LINC00116*, *PLIN1*, *SORT1*, *TRIB1*, *CIITA*, *CFTR*, *NLRC5*	Introduction of small deletions to disrupt genes in human embryonic stem (hES) cells	31
HPRT	Introduction of small deletions to disrupt gene in human induced pluripotent stem (iPS) cells	115
α1-antitrypsin (*A1AT*)	Knockin *piggyBac* Puro-TK cassette to correct a mutation in human iPS cells	23
COL7A1	Knockin loxP-flanked Puro-resistance cassette to correct a point mutation in fibroblasts and subsequently reprogrammed into human iPS cells	98
MBD3	Knockin loxP-flanked Neo-resistance cassette to disrupt *MBD3* gene in hES cells	108
β-Globin (*HBB*)	Knockin loxP-flanked Puro- or Neo-resistance cassette to correct *HBB* gene in human iPS cells	80
NPC1	Knockin *piggyBac* Puro-TK cassette to correct a mutation in human iPS cells.	83
AAVS1	Knockin rtTA and Cas9 expression cassette for Dox-inducible expression of Cas9 in hES cells	48
F8	140-kb inversion by two pairs of TALENs	102
CCR5	Knockin *piggyBac* Puro-TK cassette to introduce a 32-bp deletion in human iPS cells	154
DMD	Introduction of small indels to correct frame-shift mutation or knockin loxP-flanked Hygro-resistance cassette to correct in human iPS cells	73
miR-21, *miR-9-2*, or *TAT*	Knockin ssODN (single-strand oligonucleotide) to induce large deletions in human iPS cells	144
H11 safe-harbor locus on chromosome 0.22	Knockin GFP and NeoR genes with phiC31 attP and Bxb1 attP sites for site-specific integration in human iPS cells	158
SCN1A	Knockin loxP-flanked Neo-resistance cassette to correct a point mutation in human iPS cells	21

straightforward DNA decipher code simplifies the assembly of customized nucleases (25, 53). Similar to ZFNs, a custom-engineered TALE DNA binding domain was conjugated with a FokI nuclease domain. With this TALE nuclease (TALEN) system, human endogenous *NTF3* and *CCR5* genes were edited in the human K562 cell line (91) and other cell lines. The effectiveness of TALENs has also been demonstrated in human ES/iPS cells (31, 53) (**Table 2**).

CRISPR-Cas9 System

In some bacteria and archaea, an RNA-mediated adaptive defense mechanism, named the CRISPR (clustered regularly interspaced short palindromic repeat) system, exists to thwart invasions by foreign plasmid DNA or bacteriophages. The Type II CRISPR system is unique in the sense that the CRISPR-associated 9 (Cas9) protein alone, rather than as a complex of Cas proteins, can mediate DSBs when associated with CRISPR RNA (crRNA) and *trans*-activating crRNA (tracrRNA) (**Figure 2**). Moreover, DNA cleavage activity is retained when crRNA and

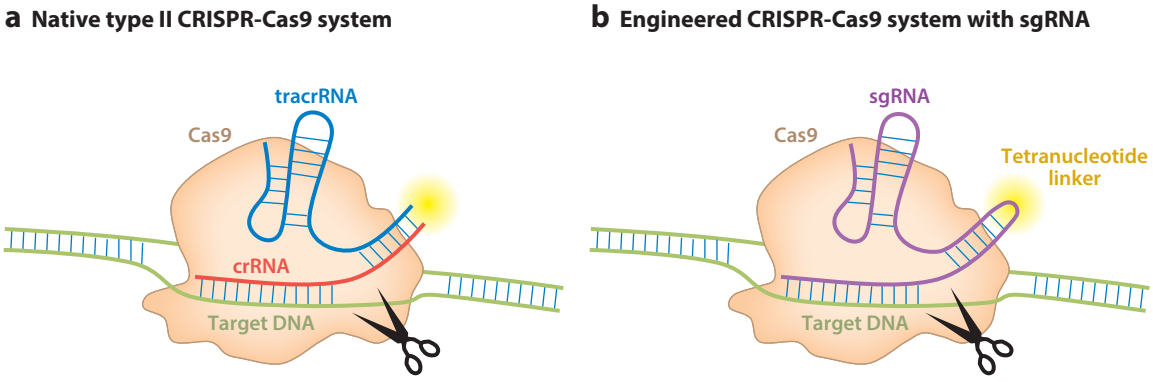

Figure 2

CRISPR-Cas9 system cleaves double-stranded DNA. (*a*) The type II CRISPR (clustered regularly interspaced short palindromic repeat) system utilizes a protein component Cas9 (CRISPR-associated 9) and two small RNAs, crRNA (CRISPR RNA) and tracrRNA (*trans* crRNA), to mediate target sequence–specific cleavage of double-stranded DNA. (*b*) To simplify expression in mammalian cells, crRNA and tracrRNA can be fused into one sgRNA (single guide RNA) by a tetranucleotide loop to generate a double-strand break at a target site.

tracrRNA are conjugated into a single guide RNA (sgRNA) (63). Remarkably, the RNA component of the CRISPR system determines the target sequence based on the Watson-Crick base pairing. Therefore, the design and construction of a target-specific sgRNA is versatile and straightforward. To adapt the prokaryotic system to work in eukaryotic cells, several groups have attached a nuclear localization signal to the Cas9 protein and have demonstrated the efficient generation of DSBs in mammalian cell lines, such as HEK293 cells (64), mouse neuro2A (N2A) cells (26), and human ES/iPS cells (32, 85). The development of the CRISPR system has caused a clear paradigm shift in the genetics field, as site-specific DSBs are now accessible for any scientist to use. Since 2013, several groups have already demonstrated the usefulness of the CRISPR-Cas9 system in pluripotent stem cells (**Table 3**).

Table 3 Examples of CRISPR-Cas9-mediated genome editing in human pluripotent stem cells

Target gene	Gene editing	Reference
AAVS1	Introduction of small indels in human induced pluripotent stem (iPS) cells	85
AKT2, *CELSR2*, *GLUT4*, *LDLR*, *LINC00116*, *SORT1*	Introduction of small deletions to disrupt genes in human embryonic stem (hES) cells	32
EMX1	Knockin ssODN (single-strand oligonucleotide) donor by double-nicking in hES cells	110
NGN3, *GATA4*, *GATA6*, *TET1*, *TET2*, *TET3*, *APOE*	Induction of small deletions or knockin ssODN donor by Dox-inducible Cas9 in hES or human iPS cells	48
CCR5	Knockin *piggyBac* Puro-TK cassette to introduce a 32-bp deletion in human iPS cells	154
HBB	Knockin *piggyBac* Puro-TK cassette to correct a mutation in human iPS cells	149
DMD	Knockin loxP-flanked Hygro-resistance cassette to correct a mutation in human iPS cells	73
METTL3	Introduction of small deletions to disrupt gene in hES cells	5
THY1	Knockin mouse *Thy1* gene into human *THY1* gene locus in human iPS cells	12
DNMT3B	Introduction of small deletions to disrupt gene in human iPS cells	55

The CRISPR-Cas9 system can be introduced into mouse embryos or those of other species directly to disrupt a target gene of interest. Gene knockout mice have become more accessible than ever before, as a single injection of Cas9 and sgRNA can induce disruption of both alleles, or a combination of multiple sgRNAs can cleave multiple genes simultaneously (143, 150). Thanks to genome-editing technologies, generating knockout animals is now expanding from mice to many other animals using in vitro fertilization (IVF). The application of genome engineering to larger animal models would open up possibilities of examining physiological functions of unexplored genes or genomic elements, modeling diseases that cannot be recapitulated in mouse models, and evaluating transplanted cells by generating immunodeficient animals as hosts.

Apart from genome editing, several groups have been investigating the modulation of gene expression or epigenetic status by taking advantage of the CRISPR system to bind to specific target sequences. For instance, a Cas9 protein lacking nuclease activity (dCas9, with two point mutations, D10A and H840A) can be targeted to a desired transcriptional region to block transcriptional initiation or elongation, which is called CRISPRi (CRISPR interference) (106). To further modulate gene transcription, dCas9 can be fused with a transcriptional repressor (such as a KRAB domain) or activator domain (such as VP16 or VP64 domain) to modulate gene transcription (22, 47, 82, 105, 132). Such artificial transcription factors can be used to construct synthetic gene circuits (156) or change cell fate, such as the myogenic conversion of fibroblasts (16).

APPLICATIONS OF GENOME ENGINEERING IN GENE THERAPY

Genomic engineering by site-specific nucleases has tremendous potential for many applications (**Figure 3**) (44, 57, 67, 116, 157). From a human health perspective, one of the most exciting applications of genome-engineering technology is in gene therapy (13, 74, 101).

Concept of Gene Therapy

Gene therapy is a way to treat diseases by altering DNA sequences and is usually applied to congenital diseases that are caused by genetic mutations. Depending on where genetic manipulations take

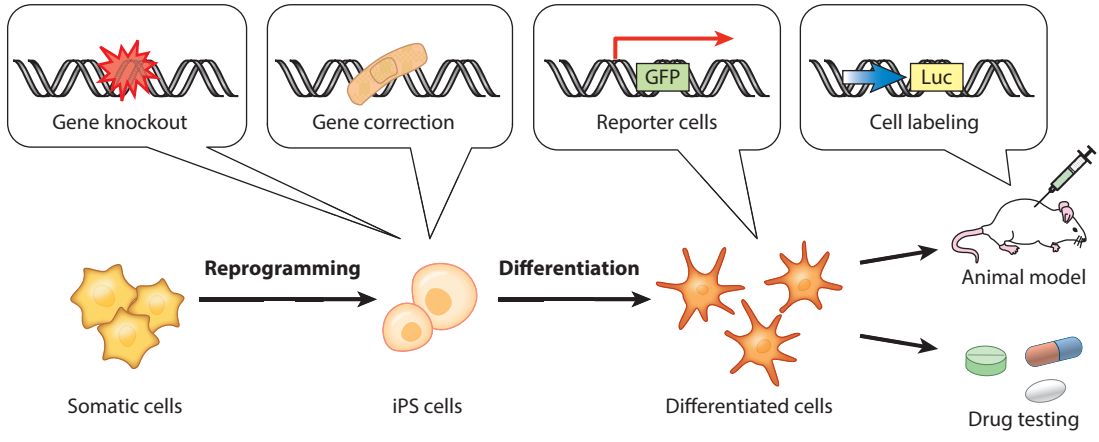

Figure 3

Applications of genetic techniques in the induced pluripotent (iPS) cell field. Genetic modification techniques have numerous possibilities for applications in the iPS cell field, such as gene knockouts for molecular studies, gene correction or introduction of mutations for disease modeling, knockin reporter cell lines for optimizing differentiation protocols, and cell labeling by reporter genes for tracking transplanted cells in animal models. Abbreviations: GFP, green fluorescent proteins; Luc, luciferase.

place, there are two methods of gene therapy: in vivo gene therapy and ex vivo gene therapy. In the latter, isolated patient cells are treated ex vivo and put back into the patient's body. The merit of ex vivo gene therapy is that it provides a quality control process that allows the confirmation of successful gene correction and the exclusion of undesired genetic mutations. Several different kinds of primary cells can be isolated from the human body; however, cells are short-lived in ex vivo culture because of senescence. Multipotent stem cells have the ability to self-renew and differentiate into several cell types. Such plasticity and self-renewing capacity is ideal for ex vivo gene therapy. In fact, hematopoietic stem cells are some of the most widely used multipotent stem cells for ex vivo gene therapy applications currently in clinical trials and have led to treatment of several severe congenital hematopoietic disorders, such as X-linked SCID, or ADA-SCID (37). Because iPS cells are pluripotent stem cells, the applicable cell types are much greater than somatic stem cells.

Gene Therapy Using Induced Pluripotent Stem Cells

In 2007, in a mouse model of sickle cell anemia, Rudolf Jaenisch's group first demonstrated that iPS cells could be used for gene therapy (17). Isolated tail tip fibroblasts were converted into iPS cells and the humanized β-globin gene was corrected by using a conventional targeting vector with short (1.7 kb) and long (7 kb) homology arms, a loxP flanked PGK-Hygromycin selection cassette, and an HSV *tk* negative selection cassette. The correction efficiency in mouse iPS cells was 1 out of 72 clones (1.4%), which was comparable with that of mouse ES cells (148). The corrected iPS cells were differentiated into hematopoietic progenitors and transplanted into sickle cell anemia model mice after irradiation to enhance engraftment of the transplanted cells. Transplanted cells contributed to the erythrocyte lineage and ameliorated the red blood defects associated with sickle cell anemia (51).

iPS cell–mediated gene therapy is promising not only for blood disorders but also for many other diseases. To advance the proof-of-principle study from mouse to human iPS cells, efficient and precise genetic manipulation by site-specific nucleases is essential to achieve successful gene correction (45, 75).

GENE CORRECTION APPROACHES

Site-Specific Knockin Gene Therapy

Retroviral vectors have been widely used in the gene therapy field; however, their genomic integration sites cannot be controlled. Unfortunately, as a result, adverse leukemic events were observed in one of the X-SCID gene therapy trials. To reduce the potential risks associated with random integration, a therapeutic gene can be inserted into a defined locus, such as the *AAVS1* locus, by using engineered nucleases via HR (52, 53). The *AAVS1* locus is considered to be one of the safe harbors that can accommodate a foreign gene without disrupting normal cell homeostasis. There are several reports utilizing this approach, such as the insertion of the α-globin gene for α-thalassemia (19), the *FANCA* gene for Fanconi anemia (111), and the *RPS19* (ribosomal protein S19) for Diamond Blackfan anemia patients (46).

The knockin approach was also used to target the transcriptional start site of the coagulation factor 9 (*F9*) gene to treat hemophilia B model mice. By introducing the *F9* cDNA sequence into the transcriptional start site, the cDNA could be expressed from its endogenous promoter. The advantage of this approach is its broad applicability regardless of the type of mutation (72).

Among knockin approaches, one interesting application would be the insertion of *Xist* RNA into the trisomy 21 chromosome. In females, one of the X chromosomes is inactivated to maintain

the dosage of X-linked gene transcripts. A long noncoding RNA on X chromosomes, *XIST*, plays a central role in inactivating one of the two X chromosomes. Epigenetically silencing one copy of chromosome 21 by ZFN-mediated insertion of the *XIST* gene at the *DYRK1A* locus of one of the trisomy 21 chromosomes has been investigated as a possible treatment for Down syndrome (62).

Chromosomal Deletion or Inversion

By using two pairs of site-specific nucleases, a 230-kb large deletion, 15-kb duplication, or inversion of up to 140 kb was possible in HEK293T cells (71). Such inversion of a large genomic region was demonstrated in the blood coagulation Factor VIII (*F8*) gene to model a mutation associated with hemophilia A in human iPS cells (102). The same technique might be used to correct *F8* inversion in patients, even though the most frequent mutation in hemophilia A is a much bigger inversion (~580 kb).

Correction of Protein Reading Frame

DNA cleavage by site-specific nucleases can induce small deletions or insertions via NHEJ or MMEJ (microhomology-mediated end joining), averaging ~10 bp in size but ranging from a single base pair to some hundreds of base pairs (4). By introducing such a small insertion or deletion within a protein coding region, some frame-shift mutations can be corrected. Duchenne muscular dystrophy is a severe muscle degeneration disease caused mainly by the truncation of dystrophin protein, due to out-of-frame mutations. We and others have demonstrated that engineering with TALENs (99) or CRISPRs (73) can restore the protein reading frame by creating a new insertion or by promoting exon skipping. Although this approach is limited to structural proteins that can tolerate minor alterations of amino acid sequences, eliminating the targeting donor template simplifies the delivery process.

piggyBac-Mediated Excision of Donor-Selection Cassette

Requirement for a drug-selection cassette is one of the limitations of gene targeting for gene therapy, as a drug-selectable gene may induce immune responses, and the insertion of an expression cassette may disrupt the endogenous gene expression pattern. In this regard, flanking the selection cassette with two loxP sites is a widely used approach to excise the selection cassette by *Cre* recombinase, although a single loxP site remains within the genomic DNA even after excision. Dr. Kosuke Yusa and colleagues (155) applied an elegant strategy to excise the entire expression cassette by using *piggyBac* DNA transposase–mediated excision, as transposition of *piggyBac* DNA transposon is footprint free (89) (**Figure 1**). With the use of ZFN-mediated targeting and *piggyBac* donor excision, a single point mutation of the α1-antitrypsin (*A1ATD*) gene in human iPS cells has been seamlessly corrected (155); however, two rounds of selection and subcloning were required. Similar approaches have been applied for correcting an HBB mutation in β-thalassemia (149), for correcting the *NPC1* mutation in Niemann-Pick Type C disease (83), and for conferring resistance to HIV by introducing a naturally occurring 32-bp deletion in the *CCR5* gene (154), as described in greater detail below.

Single-Strand Oligonucleotide–Mediated Targeting

Owing to the high HR efficiency of genome editing by a site-specific nuclease, an HR donor template can be supplied as a single-strand oligonucleotide (ssODN) (20, 107) (**Figure 1**).

ssODN-mediated targeting is a promising method for gene correction applications because custom synthesis of oligonucleotides is well-established. However, current ssODN-mediated targeting methods stimulated by a DSB still suffer from low frequency (152). Therefore, highly sensitive or robust screening methods are required to identify correctly targeted clones (79, 93). Interestingly, combination of DNA nicking by D10A Cas9 and suppression of BRCA2 is shown to enhance ssODN-mediated targeting in 293T cells (28a). Given that DNA nicks are predominantly repaired by the high-fidelity base excision repair pathway, DNA nicking is preferred as a means to maintain genomic integrity (30a). Although single nicking alone was insufficient for inducing ssODN-mediated editing (110), in combination with BRCA2 knockdown or other means, it may enhance the efficiency in human pluripotent stem cells.

CLINICAL APPLICATIONS

Clinical Applications of Site-Specific Nucleases

In the field of gene therapy research, it has long been desired to correct genetic mutations to cure genetic diseases. The development of more efficient genome-editing tools has opened up possibilities of precise gene correction in patients (74).

One unique gene therapy approach employing a site-specific nuclease was developed to treat HIV by disrupting the *CCR5* gene. *CCR5* is a coreceptor of HIV to infect $CD4^+$ T cells. Therefore, disruption of *CCR5* in T cells or their progenitor cells would lead to resistance against HIV infection (54, 84, 104). A phase I clinical trial was initiated in February 2009, and $CD4^+$ T cells from HIV patients were treated by adenoviral delivery of ZFNs targeting the *CCR5* gene. The clinical report stated that the procedures appeared to be safe and tolerated during the evaluation period (36 weeks) (133). Encouragingly, HIV genomic RNA became undetectable in one of the treated patients, although later it became apparent that this may have been due to a 32-bp deletion of the *CCR5* gene (Δ32) in one allele of the patient from the beginning, suggesting that the ZFN treatment induced the deletion on the other allele. This emphasizes the importance of homogeneous disruption of the *CCR5* gene to achieve successful treatment. In addition, because the survival time of the modified T cells is only four to six months, the current treatment protocol is temporary. This is ideal for initial safety evaluations, but the ultimate goal is to treat hematopoietic stem cells that are sustainable for the lifetime of the patient (54). Further studies will reveal the feasibility and effectiveness of this gene knockout strategy for the treatment of HIV/AIDS.

Clinical Applications of Pluripotent Stem Cells

Organ transplantation is an established medical treatment to replace damaged tissues; however, a shortage of donors is a big issue throughout the world. If one can differentiate human pluripotent cells into a functional tissue or organ, it has the enormous potential to be used as a source for transplantation. In fact, there are already some clinical trials that have been conducted using both human ES cells and iPS cells (**Figure 4**).

Clinical Applications of Human Embryonic Stem Cells

The world's first phase I clinical trial using hES cells was aimed to treat a subacute stage (7–14 days post injury) of patients with spinal cord injuries (**Figure 4**). In October 2010, oligodendrocyte progenitor cells derived from H1 human ES cells, termed GRNOPC1, were injected at the site of spinal cord damage (ClinicalTrials.gov ID: NCT01217008). Unfortunately, the trial was terminated in July 2013 after the biotech company Geron, which led the study, decided to withdraw

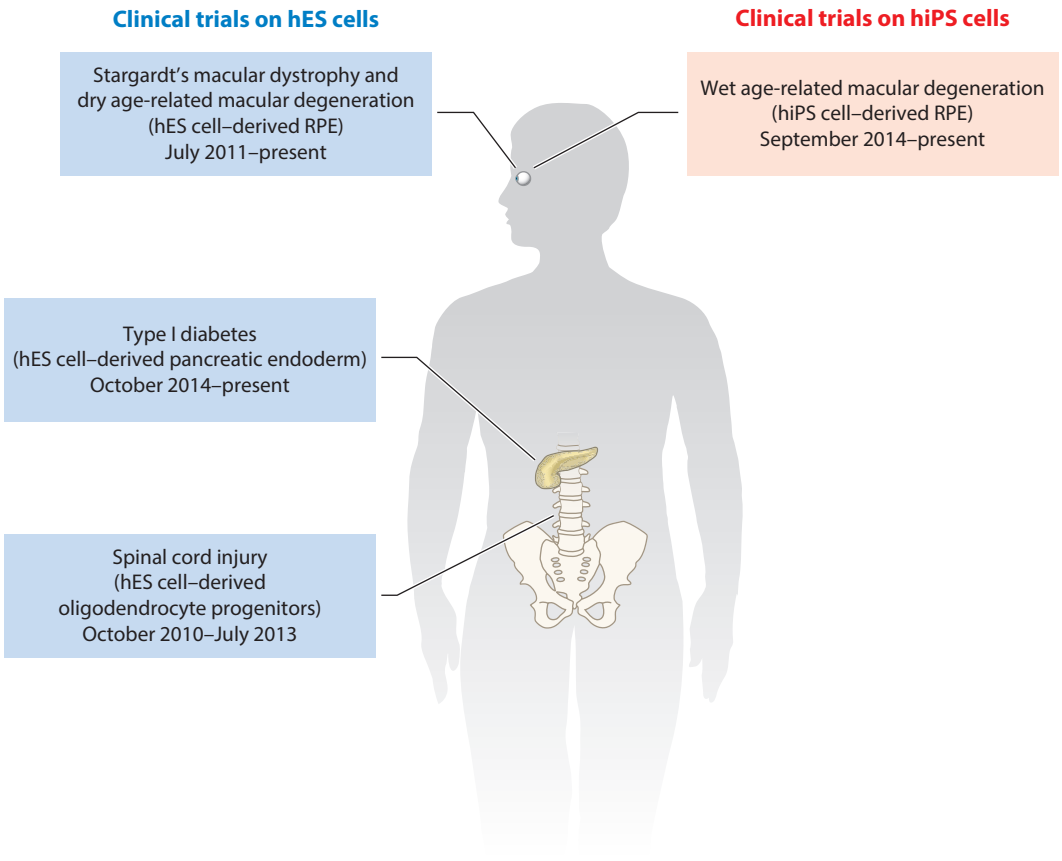

Figure 4

Clinical trials using embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. Clinical trials using human ES (hES) cells are listed on the left side, and the first human iPS (hiPS) cell–mediated clinical trial is listed on the right side. Abbreviation: RPE, retinal pigment epithelium.

from the stem cell business. So far, five patients have been treated and no major adverse effects have been reported except for minor symptoms related to the use of the immunosuppressor Tacrolimus. Details of the clinical evaluation are awaiting publication.

The second clinical trial investigated Stargardt's macular dystrophy (SMD) (NCT01345006) and advanced dry age-related macular degeneration (Dry AMD) (NCT01344993) by using retinal pigment epithelium (RPE) cells derived from a human ES cell line MA09. Dry AMD is caused by the age-related loss of RPE cells that form the foundation for supporting photoreceptor cells in the macula. SMD is a genetic disease mainly caused by mutations in *ABCA4* or *ELOVL4* genes that are important for making photoreceptor cells in the retina. Because the original ES cells retain normal *ABCA4* and *ELOVL4* genes and do not carry SMD-related genetic mutations, RPE cells derived from such ES cells are expected to be functional.

The first patients for each trial were treated in July 2011. As a phase I study to test feasibility and safety, there were no signs of tumorigenicity or apparent rejection. Out of two (SMD and Dry AMD) patients, visual improvement in the operated eye of the SMD patient was encouraging news (117). Follow-up studies are in progress (NCT01469832, NCT01674829, and NCT01691261).

The third example of an ES cell–related clinical trial is for type I diabetes. Pancreatic precursor cells (called PEC-01 cells) from CyT203 human ES cells (70) were encapsulated into a device, Encaptra, and transplanted into a patient in October 2014 as a phase I/II clinical trial (NCT02239354). Encapsulation by a semipermeable membrane is an interesting approach to prevent translocation of transplanted cells, as well as to protect them from immune responses. Again, publication of the details is awaited.

In all trials, transplantation of human ES cell–derived products was allogenic and has been conducted without matching HLA (human leukocyte antigen) types (**Figure 4**). Therefore, the current applications are mainly limited to immune privileged tissues, such as eyes and spinal cord. Nevertheless, close monitoring of immune rejection and long-term follow up is important to conclude that there was successful engraftment.

Clinical Applications of Induced Pluripotent Stem Cells

iPS cells, however, have been investigated in an autologous transplantation setting. In September 2014, the first transplantation of RPE cells derived from iPS cells (65) for Wet AMD was conducted by Dr. Masayo Takahashi's group at RIKEN CDB in Japan (UMIN ID: UMIN000011929) (**Figure 4**). It has been almost seven years since the first publication of human iPS cells in 2007. Considering that human ES cells took almost 12 years from the first establishment to the first transplantation into a spinal cord injury patient in October 2010, this was a relatively quick transition from bench to bedside. This was made possible because of the extensive studies in ES cells paving the way for iPS cells.

Because iPS cells can be generated from essentially anyone, autologous transplantation is ideal to minimize immune rejection (**Figure 5**). However, considering the burden of generating and characterizing clinical-grade iPS cells, treatment costs will be significantly increased. Therefore, allogenic transplantation, rather than autologous transplantation, might be preferred for immune-privileged tissues. HLA matching and ABO blood-type matching are established criteria for kidney or pancreas transplantation for preventing immune rejection. Therefore, it is worthwhile to match the HLA type for iPS cell transplantation to minimize the dose of immunosuppressing agents.

REMAINING CHALLENGES ON INDUCED PLURIPOTENT STEM CELLS

Large-Scale Cell Production

Although ES/iPS cells have hyperproliferative activity, differentiated cells normally do not. To yield a sufficient cell number for treatment, large-scale culture systems and efficient differentiation protocols are needed.

The eye is a relatively small organ in the human body. For the human iPS cell phase I study for RPE transplantation, 5×10^4 cells (for human ES cell trial) or an RPE sheet (i.e., 1.3×3 mm square) was prepared and transplanted. However, to treat other tissues, a higher cell number is necessary. How to expand the cells without compromising cell quality and purity is an important challenge.

Progenitor Versus Terminally Differentiated Cells

In this regard, the cell transplantation stage is another important factor to consider. Terminally differentiated cells are functional, but they do not proliferate. Once they reach life expectancy,

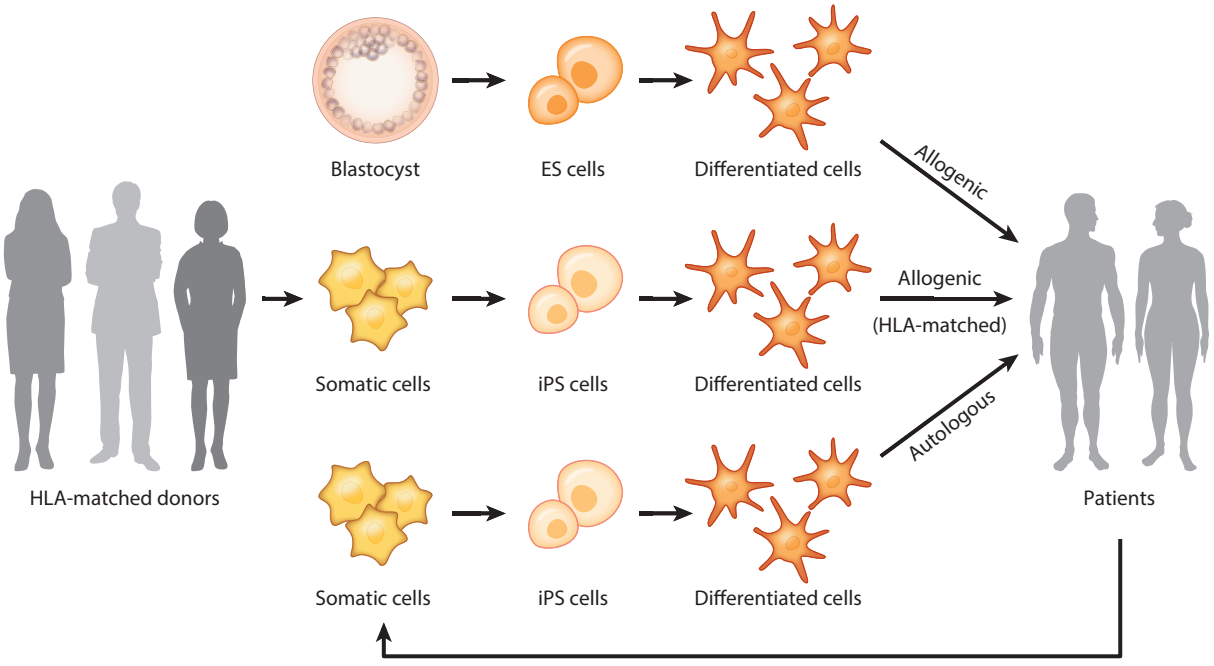

Figure 5

Schemes for pluripotent stem cell–mediated cell therapy. Currently, three main schemes have been investigated for cell therapy using human pluripotent stem cells. Human embryonic stem (ES) cells are derived from blastocysts; this process is limited in availability because of ethical issues. Therefore, cell therapies are conducted without human leukocyte antigen (HLA) matching to target immune privileged sites. Human induced pluripotent stem (iPS) cells, however, can be used in autologous or HLA-matched setting situations.

transplanted cells are eliminated. However, progenitor cells or tissue stem cells are an attractive cell source for transplantation, as they might be able to continuously self-renew and provide differentiated cells. On the basis of therapy strategies of disease or nature of cell types, the best cell types must be determined.

Differentiation Toward Particular Cell Types and Reporter Cell Lines

The differentiation of human pluripotent stem cells toward a particular cell lineage of interest is a critical step in the stem cell field; however, it requires extensive and laborious optimization of the differentiation conditions (146). Additionally, there are some cell lineages that are still difficult to differentiate from pluripotent stem cells (i.e., functional germ cells or transplantable hematopoietic stem cells by full in vitro differentiation). To aid the optimization process, visualization of the spatiotemporal regulation of a gene is useful to optimize the treatment conditions or to enrich the target cells of interest (56). To achieve these goals, a reporter gene, such as GFP, can be inserted at the translational start site or immediately after the gene (before the poly-A signal) connected by an IRES (internal ribosome entry site) or a 2A self-cleavage peptide. This way, the reporter gene reflects the endogenous expression pattern of the targeted gene. For example, pluripotent stem cell–specific genes *OCT3/4* (162) and *REX1* (6), as well as several lineage-specific genes—such as *MIXL1* to mark primitive streak-like cells (29), *OLIG2* for neuroglial cells, *FEZF2* (*FEZL*) for

corticospinal neurons (114), and *OSR1* for the intermediate mesoderm (81)—have been targeted. In addition, the *LGR5* gene was targeted to isolate adult intestinal stem cells (40).

Cell Maturation Stage

The differentiation process is considered to mimic developmental processes. Therefore, most of the differentiated cells from ES/iPS cells tend to be a reflection of the early stage of development (i.e., embryonic or infant stage). Such immature cells significantly differ from adult cells. Establishment of mature cells is important for obtaining functional cells for cell therapy.

It is noteworthy that Dr. Hongkui Deng's group reported that the addition of maturation factors *ATF5*, *PROX1*, and *CEBPA*, together with a combination of *HNF1A*, *HNF4A*, and *HNF6*, converted fibroblasts directly into mature hepatocytes with drug metabolic function (34). The maturation factors were identified from a comparative analysis between freshly isolated primary human hepatocytes and fetal liver cells. Such adult stage–specific transcription factors might exist for other lineages as well, perhaps even for ES/iPS cell differentiation processes. Further investigations are necessary to uncover these factors.

Targeting Specificity

From a site-specific nuclease point of view, targeting specificity is a major concern, particularly for clinical applications. In fact, some groups have reported high off-target mutagenesis rates of the CRISPR system in human cancer cell lines (42, 58, 77). Whole-genome sequencing (69, 120, 126, 142, 151), or exome sequencing (73, 155), is one way to investigate the risk of off-target mutagenesis. So far, several groups have investigated the nuclease-treated clones using this approach and found no significant increase in mutation events. Capture sequencing of the locus targeted by the DSB is another option to detect low frequency off-target mutagenesis (41, 138). Regarding iPS cells, several subclones should be screened to isolate appropriate clones with minimal off-target mutations. Discrimination of off-target mutagenesis from spontaneous mutations or sequence errors is still technically challenging, and further investigation is required.

CONCLUSIONS

The rapid development of iPS cell and genome-editing technologies has broadened our ability to study genomics in human cells. Applications of such genome-editing techniques are broad, encompassing areas ranging from functional studies, disease modeling, and the generation of reporter lines, to gene correction. Gene knockout studies may reveal fundamental functions of genes in human cells, and disease modeling is expected to elucidate disease mechanisms. Finally, reporter lines aid the optimization of differentiation protocols to obtain specialized cell types. Altogether, gene correction is an important tool for disease modeling and may also hold the key to gene therapy.

The year 2014 marked the arrival of iPS cells onto the clinical stage, and this is just the beginning. Further efforts are needed to tap the full potential of iPS cell–mediated cell therapy to benefit human health. In addition, incorporating newly emerging genome-editing technologies might trigger a new era of gene therapy using iPS cells.

DISCLOSURE STATEMENT

Shinya Yamanaka is a scientific advisor, without salary, of iPS Academia Japan.

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