CRISPR/Cas9 Genome-Editing System in Human Stem Cells: Current Status and Future Prospects

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Genome-editing involves the insertion, deletion, or replacement of DNA in the genome of a living organism using "molecular scissors." Traditional genome editing with engineered nucleases for human stem cells is limited by its low efficiency, high cost, and poor specificity. The CRISPR system has recently emerged as a powerful gene manipulation technique with advantages of high editing efficiency and low cost. Although this technique offers huge potential for gene manipulation in various organisms ranging from prokaryotes to higher mammals, there remain many challenges in human stem cell research. In this review, we highlight the basic biology and application of the CRISPR/Cas9 system in current human stem cell research, discuss its advantages and challenges, and debate the future prospects for human stem cells in regenerative medicine.

Background

CRISPR is a short and repeating nucleotide initially found in the genome of bacteria and archaea, and it functions to eliminate exogenous genetic elements (EGEs) that combine with Cas proteins.[1,2] Three steps are required to eliminate EGEs: new spacer integration, CRISPR/Cas combination, and degeneration (Figure 1). First, some short nucleotides of invaders may integrate with the CRISPR loci of the host as new spacers. The CRISPR RNAs (crRNAs) are then transcribed to generate a crRNA/Cas complex. Finally, the EGEs will be inactivated by the complexes under the base complementation pairing rule. To date, three types of CRISPR-Cas immune system have been identified with different CRISPR repeat sequences and Cas protein profile.[3–5] Among them, the type II CRISPR-Cas9 immune system offers strong potential in developing a totally novel genome-editing tool for biological and medical study because it utilizes RNase III in crRNA transcription and requires just one Cas9 protein to form a crRNA/Cas9 complex.[5] Almendros's group[6] identified the conservative NGG motif, proto-spacer adjacent motif (PAM), after comparing the CRISPR array of *Streptococcus pyogenes*. In 2013, two articles revealed a new genome-editing stage for biological and biomedical studies.[7,8]

Principle

Guide RNA (gRNA) and Cas9 nuclease are core components of the CRISPR/Cas9 genome-editing system (Figure 2). Unlike the original type II CRISPR-Cas immune system, its crRNA is eventually simplified to the shortest mature and functional structure without the original crRNA processing.[7,8] This simplified gRNA contains two parts: a variable region and a basic scaffold. The former normally is composed of 18–20 nucleotides that can bind the target DNA according to the base complementation pairing rule. The latter is a long scaffold-like RNA used to bind Cas9 nuclease and form a gRNA/Cas9 complex. Then, the genome-editing system still requires three nucleotides containing a PAM, NGG.[6] Only the genome DNA that contains PAMs can be identified and bound by the gRNA/Cas9 complex to generate double-strand breaks (DSBs).

Advantages

The CRIPSR/Cas9 genome-editing system offers several advantages over the zinc-finger nucleases (ZFNs) and transcription activator-like effector nuclease (TALEN) in human pluripotent stem cells (PSCs) and somatic stem cells (SSCs). First, CRISPR/Cas9 is more user-friendly than ZNF and TALEN. Several gRNAs such as synthesizing primers need to be selected for using CRISPR/Cas9, because its specificity is related only to ribonucleotide complex formation.[7,8] Second, CRISPR/Cas9 is more economical because there is little associated cost for plasmid-mediated CRISPR/Cas9. Third, as the fastest currently available genome-editing technique, genome editing using CRISPR/Cas9 can typically be achieved in 2 weeks.[9] Finally, CRISPR/Cas9 shows a higher editing efficiency than TALEN in human stem cells. Ding et al.[10] demonstrated the highest genome-editing efficiency of 79% using CRISPR/Cas9 to edit human PSCs.
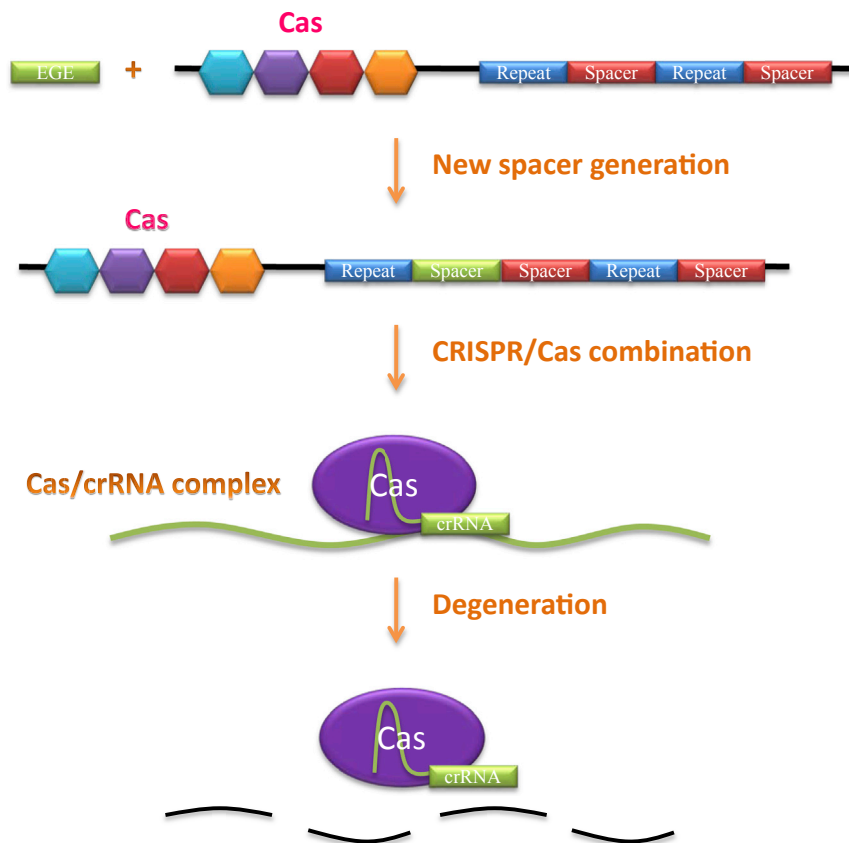
Applications in Stem Cell Studies

CRISPR/Cas9-based gene manipulation, for example, gene knockout, gene knockin, gene interference or activation, and other chromosome-related applications, has been widely utilized in biological and biomedical research.[11] Stem cells are indispensable for repair and maintenance of homeostasis. In terms of tissue repair and regeneration capacity, stem cell-mediated cell therapy and gene therapy are regarded as core components of human medicine. Inordinate manpower and financial resources have been poured into stem cell-related studies with many achievements. To date, several types of

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Figure 1. Overview of CRISPR-Cas Immune System
When EGEs invade the host, some fragments will integrate into the CRISPR loci as a new spacer casually that is co-expressed with Cas nucleases to form Cas/crRNA complexes. These complexes can identify and bind with the same EGEs during a subsequent invasion following the base complementation pairing rule and then finally break the EGEs.

finally promoted lineage differentiation. Wang et al.[14] identified the essential role of P53 in mes-endodermal differentiation through activation of the Wnt3 pathway in human ESCs. Some inducible gene knockout systems that can knock out single or multiple genes at all the stages of cell differentiation have recently been developed in human PSCs.[15,16]

Controlling Expression. Although genome-editing-based gene knockout offers a mean by which to study gene function, a less complex method is sometimes required. "Dead" Cas9 (dCas9) is a variant of Cas9 nuclease whose endonucleolytic activity has been removed. It nonetheless retains the capacity to generate the gRNA/Cas9 complex for binding with the targeted DNA regions.[17] Kearns et al.[18] fused different effector domains (VP64 or KRAB) to the dCas9 and provided a platform to control gene expression (transcription repression or activation, CRISPRi/a) in human ESCs. In addition to straightforward controlling styles, an inducible CRISPRi/a has been developed for human stem cell research. Mandegar et al.[19] fused a doxycycline-inducible dCas9 with KRAB and achieved conditional and reversible interference in human induced PSCs (iPSCs) and its derived somatic cells such as cardiac progenitors, cardiomyocytes, and T lymphocytes. Guo et al.[20] designed a doxycycline-inducible dCas9-VPR cassette to activate gene expression in human PSCs. These CRISPRi/a platforms should provide a more convenient strategy to explore gene functions and signaling pathways in human stem cell research, which are faster, more convenient, and more economical than other techniques such as RNAi and gene overexpression.[21] To achieve this, it is necessary to ligate primer-like gRNAs into plasmids to achieve rapid transcription activation or suppression without setting various controls or cDNA cloning, both of which are time consuming in RNAi or gene overexpression. For example, Liu et al.[22] utilized CRISPRi to interrupt the expression of long non-coding RNA (lncRNA) *LOC646329* to reveal its key function in the proliferation of radial glial cells. Luo et al.[23] found that suppression of miR-199a/214 cluster could significantly increase the tumor tropism in human iPSC-derived neural stem cells (NSCs). Similarly, CRISPRa may improve survivability of stem cells by controlling their gene expression following transplantation.[24] CRISPRi/a is highly specific in targeting human stem cells. But

stem cells have been approved for clinical treatments, and many have demonstrated remarkable outcomes in clinical trials.[12] Increasing reports confirm that CRISPR/Cas9 genome editing is a powerful tool that can promote stem cell research, from basic biological to translational studies (Table 1).

Basic Biological Studies

Gene Knockout. The basic biology of PSCs has always been a fundamental component of stem cell and human developmental research. In addition, the high differentiation capacity of human PSCs enables their broad application. It is therefore essential to explore the intrinsic connection between the upstream regulatory mechanism and the downstream biological features. This can be achieved by adopting a classic gene knockout strategy. CRISPR/Cas9 can be used to rapidly induce gene mutations in human PSCs without changing their genetic background, making CRISPR/Cas9 a superior technique to other gene-interfering tools (ZFN, TALEN, and RNAi).[9] When a cell's genome DNA is broken by a gRNA/Cas9 complex, the genome repair system is activated. One such system is non-homologous end joining (NHEJ). This will directly ligate the broken DNA and result in the chance of introducing a wrong base-pair deletion or insertion for gene knockout.[9] Batista et al.[13] used CRISPR/Cas9 to knock out *Mettl3* in order to erase m6A modification in human embryonic stem cells (ESCs), which prevented the self-renewal of ESCs and
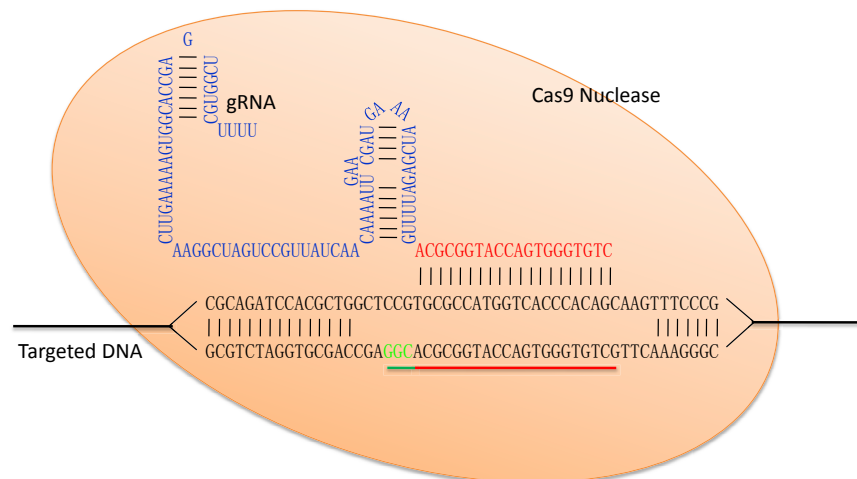
Figure 2. Schemata of CRISPR/Cas9 Genome-Editing System

gRNA contains a variable region (red nucleotides) and a basic scaffold structure (blue nucleotides). The former is complementary with one strand of targeted genome DNA that should be adjacent to PAM (NGG, green one). The basic scaffold nucleotide is then targeted to combine and support Cas9 nuclease to generate gRNA/Cas9 complexes. Finally, the targeted genome DNA will be cut at the third nucleotide from PAM (between G and C).

because it targets transcription, it cannot be utilized in the study of alternative splicing.

Genome-wide Screening. CRISPR/Cas9 has been used for genome-wide and high-throughput genetic screening in mammalian cells.[25,26] gRNA libraries have been generated to provide large volumes of genes for analyzing results through sequencing data collection and offer an alternative to the traditional RNAi library. Nonetheless, there may be differences in the response of the gene regulatory network, because RNAi knocks down gene expression at the mRNA level, whereas CRISPR/Cas9 targets gene knockout or transcription inhibition.[27] Technically, a virus mixture that contains thousands of gRNAs is produced to co-infect human stem cells with Cas9- or dCas9-expressed virus to simultaneously target thousands of genes to screen for those of interest. CRISPR/Cas9-mediated genome-wide screening strongly supports the basic biological research of human stem cells. Shalem et al.[28] built a CRISPR/Cas9 lentiviral library to simultaneously knock out 18,080 genes in human ESCs and identified many essential genes that are involved in cell survival. Liu et al.[29] identified 326 functional loci of lncRNAs in human iPSCs using the CRISPRi lentivirus library. It is clear that this genetic screening platform has the potential to reveal novel functional genes and a particular signaling pathway,[30] and should be a powerful platform for stem cell research.

Gene Knockin. Specific markers are extremely important for stem cell research and indispensable during stem cell differentiation, transplantation in vivo, and in vitro tracing. Normally, identification of a specific marker requires substantial time and energy, but CRISPR/Cas9 has enabled vast improvements. It is now possible to easily and rapidly create a specific marker by fusing epitope tags or fluorescent proteins to the genes of interest.[31,32] With the exception of NHEJ, homology-directed repair (HDR) is another mechanism by which exogenous nucleotide sequences can be introduced into the genome through homologous recombination.[33] DSBs caused by CRISPR/Cas9 significantly promote the homologous recombination between a targeted genome and exogenous DNA fragments to activate HDR

at a cell level.[9,34] For stem cell differentiation, Adkar et al.[35] used CRISPR/Cas9 to generate a COL2A1-GFP reporter human iPSC line to differentiate and purify chondrogenic cells by fusing a GFP to COL2A1, which can co-express GFP protein with COL2A1 protein. Similarly, Hunt et al.[36] built a DARPP-32-GFP cell line to monitor the pharmacological profile of striatal cultures in human medium spiny neurons (MSNs).

Other Applications. Genomic imaging is indispensable to genetic and cell biology study. CRISPR/Cas9 can also be used to visually monitor the status of the genome during the cell cycle. Chen et al.[37] modified the original nucleotide sequences of gRNA scaffold to improve its binding ability with genome DNA, and fused the GFP to the C terminus of dCas9 nuclease to finally form a live image to monitor the dynamics of the genome in the cell cycle. This technique can image fixed or living cells with single or multiple colors. It may also be used to monitor the genomic activity of stem cells. Anton et al.[38] imaged the chromatin of mouse ESCs using this method.

Disease Model and Drug Screening

Traditional animal disease models are important to human biological and biomedical studies. Nonetheless, it is difficult to simulate the real situation in a human body because of species differences. Human PSCs can overcome this limitation. CRISPR/Cas9 helps to easily and rapidly introduce a gene mutation into human stem cells to model a human disease, and avoids ethical problems and those associated with species differences. Indeed, the CRISPR/cas9 system has been shown to be an effective method for drug screening in human stem cell disease models (Table 1).

Human PSCs. Human PSCs, including ESCs and iPSCs, are ideal targets for gene knockout because they are easily expanded for further experiments from a single cell culture after resistance selection without losing their pluripotency.[9] One straightforward strategy is to knock out disease-relevant genes in human PSCs using CRISPR/Cas9 in order to explore the pathogenic mechanism in the derived cells (somatic cells, SSCs, and even tumor cells) and for drug screening. *Rb1* is known to initiate retinoblastoma in humans.[39] *RB1* null human ESCs generated by CRISPR/Cas9 showed aberrant mitochondria and were shown by drug screening to be sensitive to

Table 1. Summary of CRISPR/Cas9 Utilized in Human Stem Cell Research

	Cell Type	Function	Year	References
Basic Biological Studies				
Gene knockout	ESCs	*Mettl3*erase m6A modification leading to cell differentiation	2014	[13]
	ESCs	p53/63/73 knockout causes mesendodermal differentiation	2017	[14]
Control expression	ESCs	downregulation of Oct4 leads to lineage differentiation	2014	[18]
	iPSCs	Dox-inducible CRISPRi represses gene expression	2016	[19]
	PSCs	Dox-inducible CRISPRa activates gene expression	2017	[20]
	NSCs	inhibition of miR-199a/214 increases tumor tropism	2016	[23]
Genome scale screening	ESCs	identification of essential genes for cell survival by knockout of 18,080 genes simultaneously	2014	[28]
	iPSCs	identification of 326 functional loci of lncRNAs	2017	[29]
Gene knockin	iPSCs	generation of collagen-GFP reporter for cell sorting	2016	[35]
	ESCs	monitor pharmacological profiles of striatal cultures using DARPP-32-GFP reporter cell	2017	[36]
Clinical Translational Studies				
Disease model and drug screening	ESCs	*RB1*-null cells showed aberrant mitochondria and were sensitive to carboplatin (retinoblastoma)	2017	[40]
	iPSCs	isogenic iPSCs model for pathomechanism and drug screening (myelodysplastic syndrome)	2016	[42]
	iPSCs	point mutation iPSCs model for studying individual difference in hypoglycemia	2017	[43]
	ESCs	WRN-null hESCs model Werner syndrome (WS-iPSCs showed abnormal karyotypes seriously)	2015	[44]
	organoids	gene knockout kidney organoids showed cyst of tubules	2015	[45]
	organoids	modeling dyskeratosis congenita reveals the therapeutic functions of Wnt agonists	2016	[49]
	organoids	gene knockout intestinal organoids form tumors in mice kidney after subcapsule injection	2015	[51]
	organoids	organoids model reveals the function of *APC* and *P53* in intestinal neoplasia	2015	[52]
	organoids	organoids model reveals the function of TGF-β in colorectal cancer (CRC) formation	2016	[53]
	organoids	combinatorial drug responses in organoids model (colorectal cancer [CRC])	2016	[54]
Gene correction therapy	HSCs	gene-corrected patient HSCs showed functional recovery in X-linked chronic granulomatous	2017	[65]
	iPSCs	gene-corrected patient iPSCs recovered β-globin (HBB) expression	2015	[59]
	organoids	gene-corrected patient organoids showed functional recovery in cystic fibrosis	2013	[50]
	HSCs	gene-corrected patient HSCs recovered β-globin (HBB) expression in β-thalassemia	2016	[61]
	iPSCs	gene-corrected patient iPSCs showed functional recovery in hemophilia A	2015	[63]
	iPSCs	gene-corrected patient iPSCs showed normal phenotypes in Huntington's disease	2017	[64]
Anti-virus therapy	T cells	CXCR4-disrupted T cells showed HIV resistance	2015	[69]
	HSCs	CCR5-disrupted HSCs presented HIV resistance	2017	[70]
	iPSCs	CCR5-disrupted iPSCs and its derived blood cells showed HIV resistance	2015	[71]
	iPSCs	CCR5Δ32 iPSCs and its derived cells showed HIV resistance	2014	[72]
	iPSCs	CRISPR/Cas9-expressed iPSCs showed HIV resistance by elimination of virus RNA	2015	[74]
Anti-tumor therapy	CAR T cells	the anti-tumor efficacy of CAR T cells was enhanced through disrupting the PD-1 gene	2017	[77]
	CAR T cells	the anti-tumor efficacy of CAR T cells was improved by fusing CD19 CAR to the TRAC gene	2017	[78]
	iPSCs	NK cells derived from ADAM17-disrupted iPSCs presented higher HIV resistance	2016	[79]
hESCs, human embryonic stem cells.				

carboplatin.[40] Moreover, the isogenic human PSC knockout disease model is crucial to eliminate the individual variations and unknown gene mutations that occur during iPSC reprogramming.[41] Chang et al.[42] demonstrated different drug responses among cells derived from different patient-specific iPSCs and CRISPR/Cas9-generated isogenic PSCs. Employing CRISPR/Cas9 can precisely introduce mutations (point mutation, insertion, or deletion) into human PSCs to generate gene mutant PSCs and provide a patient disease model. It bypasses the limitations of iPSC generation that requires biopsy or re-programming, and provides a strategy to determine precisely why different patients with the same mutations present with different features during pathogenesis. Guo et al.[43] used CRISPR/Cas9 to induce a point mutation (c.1288G>T) in the *Men1* gene of normal iPSCs to study the effect of individual differences in hypoglycemia.

A CRISPR/cas9-mediated human PSC disease model can be applied when use of patient-specific iPSCs is not appropriate or possible. For example, Zhang et al.[44] used WRN null human ESCs to model Werner syndrome because patient-specific iPSCs showed abnormal karyotypes. Taken together, CRISPR/cas9 technologies employed in human PSCs can provide an array of genetically defective human disease models.[41,45] Nonetheless, it is important to bear in mind that PSC-derived somatic cells are young cells that more closely resemble fetal cells.

Human SSCs. CRISPR/Cas9 can be used to manipulate human SSCs including hematopoietic stem cells (HSCs), NSCs, and mesenchymal stem cells (MSCs).[46–48] The challenge lies in obtaining a sufficient number of pure cells for disease modeling and drug screening if these SSCs are difficult to maintain or expand from a relatively small number of cells. Use of the CRISPR/cas9 system has enabled many genes in HSCs to be successfully manipulated.[46] Unfortunately, difficulties persist in the maintenance of these gene-manipulated HSCs during selection, purification, and expansion. These limitations restrict the application of CRISPR/Cas9 in disease modeling and drug screening.

Organoids. Human organoids are 3D cultures derived from human PSCs, SSCs, or primary tissues. They have the ability of self-renewal and can self-organize to be primary tissue-like clumps that contain several kinds of cells. Hence they are regarded as better sources for biomedical research than 2D human cell cultures. PSC-derived organoids have been utilized to model human genetic disease and screen drugs with the assistance of CRISPR/Ca9-mediated gene manipulation. Basically, gene mutations are introduced into PSCs using CRISPR/Cas9; then the engineered PSCs become gene mutant organoids under 3D culturing conditions and can be used in disease modeling and drug screening. Freedman et al.[45] generated gene mutant kidney organoids from knockout podocalyxin, *PKD1* or *PKD2* genes of human ESCs using CRISPR/Cas9, which showed abnormal features (junctional organization defects of podocyte-like cells or cyst of kidney tubules). The *DKC1* gene mutation causes congenital dyskeratosis. Woo et al.[49] generated intestinal organoids from patient-specific iPSCs, both isogenic and gene-corrected organoids, to reveal a feedback loop between the Wnt pathway and telomere function. This also demonstrated the therapeutic action of Wnt agonists in the treatment of congenital dyskeratosis.[49] In addition, CRISPR/Cas9 can be utilized to directly mediate gene manipulation in SSCs or primary tissue-derived organoids. Their pluripotency can be sustained well under 3D culture conditions when screening the targeted colonies.[50] This strategy has been proposed as a new platform for cancer research. Matano et al.[51] engineered normal human intestinal organoids by knocking out several tumor suppressor genes and oncogenes simultaneously, and showed formation of tumors following subcapsular injection into the kidney. Similarly, Drost et al.[52] produced the most common mutant genes in intestinal organoids to reveal the functions of *APC* and *P53* genes in intestinal neoplasia. Fessler et al.[53] generated *BRAF*[V600E] mutant organoids to explore the function of transforming growth factor β (TGF-β) in

transforming sessile serrated adenomas (SSAs) into colorectal cancer (CRC). SSCs or primary tissue-derived organoids can also be utilized to screen anti-tumor drugs following the introduction of gene mutations by CRISPR/Cas9. Verissimo et al.[54] tested the combinatorial drug response for treating CRC using *RAS* and *KRAS* gene mutant normal and tumor organoids.

Stem Cell Therapy

DSB introduced by CRISPR/Cas9 significantly promotes HDR in targeted cells. Original stem cells can be easily remolded using these genetic engineering techniques to generate new function-modified stem cells for cell therapy. There is no doubt that CRISPR/Cas9 has significantly broadened the application of stem cells in human regenerative medicine.

Gene Correction Therapy. Many gene mutations at the genome level often result in genetic disorders. Although allografts have been widely utilized in preclinical experiments and clinical cell therapy studies, immune rejection remains a concern.[55,56] CRISPR/Cas9-mediated gene correction provides a new vision of cell therapy for many human genetic diseases. The general strategy is to break the genomic DNA near the targeted genes using CRISPR/Cas9 and simultaneously provide a donor that presents normal sequences. The mutant gene fragments can be corrected through homologous recombination, and the associated functional defect in the targeted cells is corrected. It has been demonstrated in an animal model that gene correction offers a definitive cure for genetic disease. Li's group[57] co-injected gRNA, Cas9 nuclease, and healthy donor plasmid of the *Crygc* gene into the zygotes of cataract mice and generated cataract-free mice. Patient-specific iPSCs are the most common source for gene correction because they can be differentiated into all kinds of functional somatic cells after gene correction. Gene-corrected β-thalassemia patient-specific iPSCs are able to restore HBB expression and be differentiated into functional hematopoietic progenitor cells.[58,59] Patient-specific organoids offer a promising source for gene correction and are more easily differentiated into somatic cells for transplantation compared with PSPs. Schwank et al.[50] successfully obtained function recovered cells after they repaired the mutant CFTR gene in human intestinal stem cell organoids. For therapeutic purposes, stem cells should be maintained in a "stemness" state. Hence we normally do not criticize the purity of manipulated cells, and use adeno-associated virus (AAV) to deliver the donor for gene correction. Porteus's group[60,61] finally obtained around 38% biallelic-corrected β-thalassemia HSCs using AAV-assisted gene correction after rough purification. This was sufficient for cell transplantation and avoided the difficulties caused by random integration in lentivirus or retrovirus-meditated gene therapy.[60,61] CRISPR/Cas9-mediated gene correction has become a principal therapeutic strategy in the study of genetic diseases such as β-thalassemia, Duchenne muscular dystrophy, hemophilia A, Huntington's disease, and X-linked chronic granulomatous disease.[58,62–65] It should nonetheless be noted that although we can obtain functionally recovered stem cells after gene correction in vitro, their therapeutic safety and efficacy after transplantation remain to be evaluated.

Anti-virus Therapy. Some viruses bind to the surface receptors of host cells during infection. As mentioned above, CRISPR/Cas9 can help modify the targeted cells efficiently and easily. Hence infection with HIV can be prevented by modifying the receptors of human cells. For example, CCR5 and CXCR4 are co-receptors of human white blood cells for HIV infection.[66,67] There are reports showing that CCR5- or CXCR4-disrupted human CD4^{+} T cells, HSCs or iPSCs, present HIV resistance after CRISPR/Cas9-mediated gene modification.[68–71] Nevertheless, cells acquired HIV resistance through direct gene disruption, and some basic functions may also have been affected. It may be advisable to first define some natural mutations that do not affect the host's biological functions before utilizing CRISPR/Cas9 to introduce desirable mutations (e.g., point mutation) in cell engineering. Ye et al.[72] introduced a natural mutation (CCR5Δ32) into normal human iPSCs and finally obtained HIV-resistant monocytes and macrophages after cell differentiation. In addition, CRISPR/Cas9 has been demonstrated to be effective in targeting the genetic material of viruses.[73] Liao et al.[74] engineered human iPSCs to express HIV-targeted gRNA and Cas9 protein that could protect cells from virus infection by disrupting the RNA of HIV in iPSCs and their derived adult cells.

Anti-tumor Therapy. Genetically engineered human immune cells and stem cells have already been widely used in anti-tumor research, such as delivering anti-tumor proteins and inducing cancer cell death.[75] Chimeric antigen receptor (CAR) T cells are considered a milestone in the cure of refractory blood cancer.[76] CRISPR/Cas9 can be used to increase their anti-tumor efficacy by disrupting the programmed death protein 1 (PD-1) in CAR T cells by eliminating PD-L1 (PD-1 ligand) expressed tumor xenografts in vivo.[77] In addition, CRISPR/Cas9 can be utilized to eliminate the risks associated with lentivirus-mediated CAR T cells, such as the potential of oncogenesis and recovery of replication. Eyquem et al.[78] engineered T cells to express CAR uniformly by directly fusing CD19 CAR to cells' TRAC gene and also increased their potency. For the treatment of solid tumors, Blum et al.[79] introduced the stable expression of CD16a (S197P) and disrupted ADAM17 in human iPSCs that could further differentiate into natural killer cells for anti-tumor therapeutic research.

Challenges
Off-Target
A common problem of all gene manipulation techniques, including CRISPR/Cas9 systems, is that they miss their target. The mismatch between gRNA and genomic DNA is the main cause of non-specificity in genome editing. It has been shown that Cas9 nuclease has diverse tolerance to the mismatch between gRNA and targeted genomic DNA. The PAM mismatch (e.g., RNG) can also lead to being off-target.[80,81] The ratio of being off-target is relative to the targeted genes and hosts. For example, CRISPR/Cas9 can generate off-targets at a different ratio for different genes (60% for *HBB* gene and 22% for *CCR5* gene) in the HEK293 cell line, while the ratio of off-targets is just 0.34% for the *RNF2* gene in the HeLa cell line.[82,83] Off-targets can also be identified in stem cells. Wu et al.[84] revealed that off-target

sites were mainly related to genes in mouse ESCs. Fortunately, recent whole-genome sequencing results have indicated that the incidence of off-targets caused by CRISPR/Cas9 is very low in human stem cells.[85–87] Although some inaccuracy is inevitable, it can be controlled or reduced using several common strategies.

Optimize gRNA Design. Well-designed gRNAs play very critical roles in ensuring a high efficiency of genome cleavage and low incidence of off-targets in human stem cell research. There are various online tools for gRNA design that are user-friendly.[88–94] When sequences of targeted genes are uploaded, all gRNAs that contain PAM (NGG) will be generated automatically, and the possibility of being off-targets is evaluated. Under normal circumstances, a higher score means a lower possibility. Therefore, the gRNAs with high scores are recommended in order to reduce the number of off-targets. Nevertheless, deep sequencing or nuclease detection is required to examine the incidence of off-targets in stem cells. Some studies have shown that shorter gRNAs can reduce the occurrence of off-target by reducing their mispairing with the targeted genome, but the indels of on-target will also decrease accordingly, because binding of shorter gRNAs to the targeted genome is not as stable as that of longer ones.[82,83]

Modify Cas9 Nuclease or gRNA. Cas9 nickase (Cas9n) is a mutant version of Cas9 that generates just a nick on the gRNA-bound strand without breaking the whole double-strand DNA.[95] This system needs a pair of gRNAs to form two gRNA/Cas9n complexes for DNA cleavage and can dramatically decrease the off-target in human ESCs.[96] Kleinstiver et al.[97,98] modified the traditional *Streptococcus pyogenes* Cas9 (SpCas9) to identify other PAM sequences or improve fidelity. Fu et al.[99] reduced off-targets 5,000-fold by using truncated gRNAs.

Exploit Alternatives to Classic SpCas9. Type I and type III CRISPR-Cas immune systems share a similar mechanism to type II. Tsai et al.[100] utilized a dimeric RNA to guide the highly specific cleavage of FokI nucleases based on the classic CRISPR/Cas9 system. Cas3 and Cmr are nucleases of the type I and type III CRISPR-Cas immune system and show better specificity than Cas9.[101,102] More encouragingly, no off-target has been detected using the CRISPR/Cpf1 system.[103,104]

Editing Efficiency
Editing efficiency is another important aspect of gene manipulation techniques. CRISPR/Cas9 has been widely utilized and has shown high genome-editing efficiency in various mammalian cells.[105] In addition, the high editing efficiency can significantly reduce costs when processing stem cells in vitro. Nonetheless, its genome-editing efficiency is insufficient for human stem cell research and hampers the transfer of genome editing from the bench to the bedside. For example, a virus is commonly used for CRISPR/Cas9 delivery, but this confers risks for the clinical transplantation of stem cells. It is almost impossible for some stem cells, such as HSCs and MSCs, to be expanded sufficiently for clinical application from screening a single cell after the manipulation of CRISPR/Cas9.

Optimize the Expression of gRNA/Cas9. Cas9-induced genome editing is related to gRNA expression and gRNA/Cas9 binding.[106] Employing purified gRNA and Cas9 protein has been shown to directly improve the genome-editing efficiency in human stem cells and provide more stable and sufficient Cas9 nuclease for genome editing. Kim et al.[107] delivered purified gRNA and Cas9 into human H9 ESCs, and finally achieved 23% indels, which was almost five times higher than that reported by Mali et al.[8] in 2013. Gundry et al.[108] remarkably enhanced the genome-editing efficiency of CRISP/Cas9 in human HSCs using purified gRNA and Cas9 nuclease. This avoids the effect of protein transcription and translation. In addition, editing efficiency can be improved by controlling the delivery time of gRNA and Cas9 nuclease in 293 cells, and may also be appropriate for stem cells.[109]

Modify gRNAs or Cas9 Nucleases Artificially. Longer gRNA binds to Cas9 nuclease more tightly with a consequent huge improvement in genome-editing efficiency.[105] Exogenous RNAs cannot stay long in human cells because of RNA degeneration.[110] Genome-editing efficiency can be improved if gRNA can be maintained for a longer time prior to degradation. Hendel et al.[111] utilized some chemical groups to protect the phosphoramidites of gRNAs and significantly enhanced the genome-editing efficiency of CRISPR/Cas9 in T cells and CD34^{+} HSCs. Other artificially modified Cas9 nuclease or alternatives to CRISPR/Cas9 have also been reported to have greater genome-editing efficiency, such as Cas3-CRISPR, type III-A CRISPR-Cas-Csm, FokI-CRISPR, and Cpf1-CRISPR.[100–102,104] Nonetheless, stronger experimental evidence is required to support these alternatives in stem cell studies.

Preprocess Host Cells. CRISPR/Cas9-mediated genome editing depends on NHEJ and HDR. Yu et al.[112] identified two compounds (L755507 and brefeldin A) that improved genome-editing efficiency by enhancing HDR efficiency in human PSCs. SCR7 is a small molecular inhibitor of the NHEJ pathway.[113] Zygotes treated with SCR7 showed a 19-fold improvement in gene knockin efficiency using CRISPR/Cas9.[114] Similarly, RS-1, an HDR enhancer, improved the gene knockin efficiency 2- to 5-fold in mouse embryos.[115] Other bioactive proteins, such as E1B55K and E4orf6, can likewise increase the knockin efficiency.[116] Although the toxicity of these small molecules and proteins requires extensive evaluation in human stem cells, they provide invaluable information to improve the editing efficiency of CRISPR/Cas9 in stem cell research.

Utilize Auxiliary Elements. Microhomology-mediated end joining (MMEJ) is another DNA repairing system in cells.[117] On this basis, a precise integration into a target chromosome (PITCh) system has been designed to improve the integration efficiency of gene knockin using several gRNAs to cut genome and PITCh vectors.[118] It is easier during donor construction, because the PITCh vector needs only a short micro homologous arm (5–25 bp) that can be synthesized directly. In contrast, classical homologous arms normally comprise thousands of base pairs of DNA amplified by PCR. Nonetheless, more experimental evidence is required to support its use in human stem cells. *PiggyBac* is a transposon system that can achieve change in position and insertion and deletion of DNA fragments from a chromosome.[119] It has been reported to dramatically increase CRISPR/Cas9-mediated genome-editing efficiency in human PSCs from the normal level of 1%–5% without using an integrated screening cassette.[58,120] Nonetheless, the random transposable action of *PiggyBac* transposons needs to be evaluated.

Delivery Routes

The commercial CRISPR/Cas9 genome-editing system is generally presented as plasmids, RNAs, or proteins. Nonetheless, this system cannot be used for gene manipulation unless they can be delivered into the targeted hosts. Selection of an appropriate delivery method is thus vital for CRISPR/Cas9-mediated gene manipulations. Normally, they have delivery strategies similar to other gene-manipulating tools such as RNAi, ZFN, or TALEN. Nonetheless, because of the unique features of human PSCs and SSCs in regenerative medicine, different delivery strategies need to be considered carefully.

Transient Expression Strategies. Gene manipulation can be easily achieved by transient transfections of gRNA/Cas9 nuclease-expressed plasmids or their purified synthetic products in common cells.[7,8,107,121] The situation becomes more complex in stem cell studies as stem cells are more fragile, sensitive, or resistant to the transient delivery methods than common somatic cells and immortalized cell lines. Hence appropriate delivery methods are required. In terms of efficiency of delivery, micro-injection achieves the highest up to 100% for direct injection of substrates into targeted cells one by one through glass micro-pipettes.[122] But this micro-injection cannot be applied for large-scale injections, and thus limits its application to immortalized cells (e.g., PSCs).[123] Both electrotransfection and nucleofection have achieved high transfection efficiency in various human stem cells. The former can treat more kinds of cells in principle, while the latter presents higher cell viability after transfection.[58,124–126] Millions of cells can be handled in one reaction based on electroporation; nonetheless, much time and effort have been expended in purifying the real transfected cells using selection cassettes.[58,124] Lipidosome reagents are not as popular as the other three methods for human stem cell transfection, because their transfection efficiency is poor when targeting many human stem cell lines. In addition, many human stem cell lines are extremely sensitive to the cytotoxicity of transfection reagents and the mechanical damage that occurs during cell pretreatments. Some new lipidosome-like delivery methods are now available with CRISPR/Cas9. Yin et al.[127] exploited a new lipid nano-particle to deliver Cas9 mRNA for genome editing; Jiang et al.[128] optimized the formula using lipid-like nano-particles (LLNs) for gRNA and Cas9 mRNA delivery. It has been noted that some stem cell lines are sensitive and hard to be transfected.[50]

Lentivirus-Mediated Strategy. Lentivirus is another commonly used vehicle for CRISPR/Cas9 delivery. It is more stable, more efficient, and can theoretically infect almost all kinds of human stem cells in vivo and in vitro with high efficiency and low cellular toxicity.[37,129,130] It presents several remarkable advantages for stem

cell studies compared with transient strategies. First, lentivirus can introduce high, stable, and permanent expression of exogenous genes for random genome integration, which is crucial for CRISPR imaging and CRISPR screening when infecting human stem cells such as human PSCs, SSCs, or organoids.[18,29,37,38,131] Second, CRISPR-mediated genetic screening needs lentivirus to deliver thousands of gRNAs into stem cells with high efficiency and low cellular toxicity.[25,132] Third, lentivirus is directly added for co-culturing with cells and eliminates the damage from cell pretreatment. Nonetheless, the random genome integration remains an inevitable barrier that limits clinical applications of lentivirus. Although lentivirus has been proven to be an effective delivery vehicle to mediate genome editing in stem cells, it is still not recommended for single or multiple gene manipulations.[133,134] Uncontrolled gene mutations introduced by random integrations are not desired, while integrase-defective lentivirus shows a lower infection efficiency and gene expression level than the normal lentivirus.[135]

AAV-Mediated Strategy. Theoretically, AAV supports all applications of the CRISPR/Cas9 gene manipulating system similar to lentivirus (e.g., genome editing, CRISPRi/a, CRISPR imaging, and so on) because it shares the advantages of lentivirus in stem cell research.[136,137] We can easily obtain extremely high titering AAV virus using helper plasmids during virus packaging with consequent higher infection efficiency than lentivirus.[138,139] More importantly, an AAV-delivered donor is genome integration-free. It permanently exists as episomal DNA in cells and largely promotes efficiency of CRISPR/Cas9-mediated gene correction in stem cell therapy.[140–142] For example, Tabebordbar et al.[143] repaired mutant *Dmd* genes by AAV-delivered CRISPR/Cas9 in muscle stem cells. Unfortunately, the insert size capacity of AAV was far smaller than lentivirus. With the exception of SaCas9 (*Staphylococcus aureus*), smaller nucleases need to be exploited to enhance editing efficiency when choosing AAV as the delivery vehicle.[144] In addition, AAV-delivered gRNA and Cas9 nuclease will be permanently expressed in all new divided cells. This may induce undesired genome damage and ultimately lead to cell death.[145] Although this situation has been resolved by delivering donor (AAV) and CRISPR/Cas9 (transient delivery strategies) separately, the advantages of AAV are partly concealed.[61] In addition, the immunogenicity of AAV remains another risk that must be considered.[146]

Novel Delivery Strategies. With the development of CRISPR/Cas9, some novel delivery methods have emerged that eliminate the toxic effects or safety problems associated with transient transfection or virus infections. A chip containing micro-constrictions can generate transient membrane holes on cells by mechanical deformation to deliver CRISPR/Cas9 plasmids when the mixture of cells and plasmid pass the chip rapidly.[147] This method is reported to introduce high efficiency in genome editing with low cell loss in hard-to-transfect cells.[147] Based on the membrane punching mechanism, it also has the advantages and disadvantages of electroporation. The other methods are to utilize cell-penetrating peptides (CPP) to conjugate gRNA and Cas9 nuclease for CRISPR/Cas9 delivery in a plasmid-free, transfecting reagent-free, and virus-free manner.[121] A disadvantage is its relatively low transfection efficiency.

Prospects

Since its introduction in 2013, the CRISPR/Cas9 genome-editing system has been rapidly developed and widely used in all human stem cell studies. All gene-manipulating capacities (e.g., knockout, knockin, knockdown, and expression activating) are incredibly integrated in one technique. It is user-friendly, efficacious, and economical such that genome manipulation ceases to be a challenge for new researchers. Undoubtedly, the CRISPR/Cas9 genome-editing system has revolutionarily changed the fundamental and translational stem cell research. Although solutions are still required to reduce the off-target effect, improve editing efficiency, and exploit novel delivery strategies at a low cost and with high safety for clinical stem cell studies, CRISPR/Cas9-mediated preclinical studies have made remarkable progress and offer huge potential in human stem cell and regenerative research.

AUTHOR CONTRIBUTIONS

Conceptualization, Z.Z. and Q.L.; Writing – Original Draft, Z.Z. and Q.L.; Writing – Review & Editing, Z.Z., Y.Z., F.G., S.H., and Q.L.; Supervision, K.S.C. and Q.L.; Funding Acquisition, K.S.C., H.-F.T., and Q.L.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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