Int J Clin Exp Med 2017;10(8):11409-11423
www.ijcem.com /ISSN:1940-5901/IJCEM0053428

Review Article

Comprehensive update on applications of CRISPR/Cas9 for hematological diseases

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Received March 21, 2017; Accepted April 26, 2017; Epub August 15, 2017; Published August 30, 2017

Abstract: There have been significant advances in understanding the genetics and molecular basis of a number of hematological disorders in recent years, but clinical interventions, such as supportive treatment and supplementary therapy, are quite limited and not optimal. Hematopoietic stem cell transplantation (HSCT) is currently the only cure for some hematological diseases. However, a lack of human leukocyte antigen (HLA)-matched donors as well as the fatal graft-versus-host disease (GVHD) greatly hinder the application of this therapy, apart from the huge cost for transplantation. Therefore, it is of urgent necessity to find new therapies for hematological patients, among which gene therapy is a promising one. Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) is a RNA-guided endonuclease system developed from the bacterial type II CRISPR/Cas adaptive immunity. CRISPR/Cas9 has been leading to major breakthroughs in the field of gene therapy for inherited diseases due to its unprecedented simplicity and multiplexability. Hematology is one of the first fields to benefit from gene therapy. Up to now, researchers have explored genetic manipulation in sickle cell disease, β-thalassemia anemia, hemophilia, leukemia, and Fanconi anemia. In this review, we highlight the recent progress in treating hematological disorders via CRISPR/Cas9 as well as challenges standing in the way of translating it into a clinically relevant therapy.

Keywords: CRISPR/Cas9, gene therapy, hematological disease, inherited disorder, off-target effect, delivery method

Introduction

Currently, hematopoietic stem cell transplantation (HSCT) is considered to be the only way to cure a number of hematological diseases. However, the limited chance of finding human leukocyte antigen (HLA)-matched healthy donors greatly affect the application of this therapy. Moreover, the lethal complications of HSCT, such as graft-versus-host disease (GVHD), reduce the success rate of HSCT [1]. Relapse is another critical issue to be addressed [2]. All of these conditions indicate that novel therapies are of urgent necessity.

In the field of gene therapy, scientists have made great progress during recent years. The rapid development of zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) technologies provides significant opportunities to precisely target certain genomic locus and correct the pathogenic mutations. CRISPR-associated protein 9 (Cas9) is a RNA-guided programmable DNA endonuclease from the microbial type II CRISPR/Cas adaptive immune system. Cas9 derived from *Streptococcus pyogenes* (SpCas9) is the most widely used one [3]. When guided by the 20-nt single guide RNA (sgRNA), Cas9 can induce site-specific DNA double-strand breaks (DSBs). The endogenous DNA repair system will mend the DSB via either error-prone non-homologous end joining (NHEJ) or homology-directed repair (HDR). In the presence of donor DNA, insertions, deletions, nucleotide substitutions or even genomic sequence rearrangements can be introduced into the

Table 1. Gene therapy for different hematological diseases using CRISPR/Cas9

Hematological disease	Gene	Mutation	Donor	Delivery	Model	Reference
Sickle cell disease	β-globin gene (*HBB*)	Nt. 69A > T (p.E6V)	Plasmid donor	Nucleofection	Human induced pluripotent stem cells (iPSCs)	[15]
			Plasmid donor and integrase-defective lentiviral vector (IDLV)	Electroporation and transduction	hematopoietic stem and progenitor cells (HSPCs)	[17]
			Single-strand oligodeoxynucleotides (ssODNs)	Transduction and nucleoporation	iPSCs	[16]
			Adeno-associated viral vector	Electroporation and transduction	Hematopoietic stem cells	[18]
			ssODNs	Electroporation	HSPCs	[19]
β-thalassemia anemia	*HBB* gene	-28 (A/G) mutation of the promoter and the 4-bp (TCTT) deletion at codon 41 and 42 of exon 2	*PiggyBac*-based donor	Electroporation	iPSCs	[20]
		IVS2-654 (C > T)	*piggyBac* donor vector	Electroporation	iPSCs	[21]
		CD41/42: -CTTT	ssODNs	Electroporation	iPSCs	[22]
		Homozygous 41/42 deletion	PCR products of the correct gene	Electroporation	iPSCs	[23]
		13 kb of the β-globin locus	-	Nucleofection	HSPCs	[26]
Hemophilia A	Coagulator factor VIII (*F8* gene)	Chromosomal inversion	-	Microporator system and electroporation	iPSCs	[28]
Hemophilia B	Coagulator factor IX (*F9* gene)	Nt.31094T > G (p.Y381D)	ssODNs, plasmid donor & adenoviral vector	Hydrodynamic tail vein (HTV) injection	Mouse	[4]
Leukemia	*ASXL1*	c.2128G > T, (p.G710X)	ssODNs	Chemical transfection	CML cell line KBM5	[37]
Fanconi Anemia	*FANCC*	c.456+4A > T	Donor plasmid	Chemical transfection	Patient-derived fibroblasts	[40]
	FANCI	c.1461 T > A	Donor plasmid	Electroporation	iPSCs	[42]

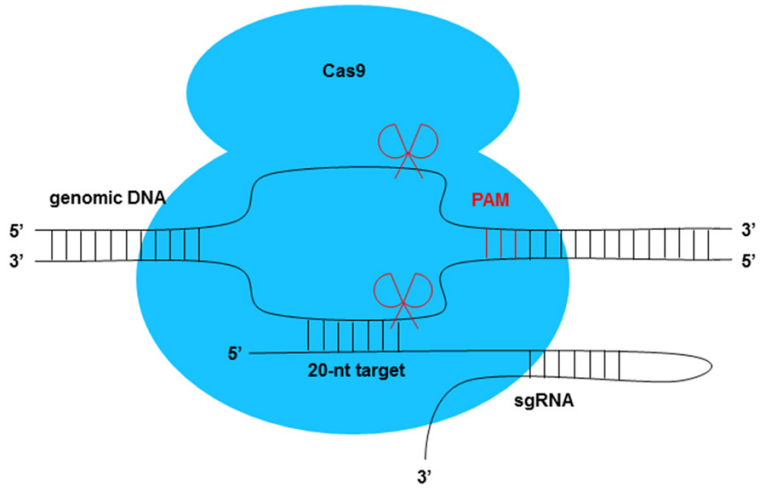

Figure 1. The CRISPR/Cas9 system. Cas9 is guided by a modified 20-nt chimeric sgRNA which encodes a sequence complementary to a target protospacer in the genome. This target site recognition process requires a PAM, which is NGG for Cas9 derived from Streptococcus pyogenes. Upon the Watson-Crick base pairing of sgRNA and its target sequence, Cas9 cuts both complementary and non-complementary strands and creates a DSB. Cas9: CRISPR-associated protein 9; PAM: protospacer adjacent motif; sgRNA: single guide RNA.

genome with unprecedented flexibility and simplicity.

The easy accessibility of hematopoietic stem cells (HSCs) creates favorable conditions for scientists to precisely correct the disease-causing mutations in patient-derived cells and then differentiate them into certain blood cells. Briefly, CRISPR/Cas9 system can be utilized to edit mutated patient-derived autologous hematopoietic stem cells in vitro, and then genotyping will be carried out to verify if HSCs are correctly edited. Corrected cells can then be re-implanted back to the patient to improve disease conditions. With the development of induced pluripotent stem cells (iPSCs), another strategy is to generate patient-specific iPSCs, correct the disease-causing mutations, differentiate them into HSCs, and then re-implant edited cells back to the affected individual. Actually, even a mild increase of some blood components is able to alleviate patients' symptoms and yield significant clinical benefits. For example, as low as 1% increase of the plasma FIX level can partially restore clotting activity [4].

With the popularization of next generation sequencing, novel causal mutations of hematological diseases are discovered and molecular diagnoses are carried out more frequently [5-7]. The pathogenic mechanisms of a growing number of diseases have been recognized at the gene level. These new advances provide a strong basis for gene therapy in hematological disorders. CRISPR/Cas9 has been investigated to treat hematological disorders such as sickle cell disease, β-thalassemia anemia, hemophilia, leukemia, and Fanconi anemia (**Table 1**), and the exciting results obtained provide us with full confidence to eradicate hematological genetic disorders. This review surveys recent advances of CRISPR/Cas9 gene therapy in hematology and discusses challenges to be tackled before applying it to clinical practices.

Development of the CRISPR/Cas9 system

Targeted nuclease systems have been widely studied during recent years, including ZFNs, TALENs and the revolutionary CRISPR/Cas9 system. A targeted nuclease creates a double strand break that can be repaired by either non-homologous end joining or homology-directed repair. While NHEJ is error-prone and easily creates genomic insertions and deletions (indels), HDR can help to precisely manipulate the genome by introducing insertions, deletions, nucleotide substitutions or even genomic sequence rearrangements in the presence of a DNA template.

The CRISPR/Cas9 system is derived from the microbial adaptive immune system which fights against virus or plasmid invasion. Cas9 is guided by a modified 20-nt chimeric sgRNA which encodes a sequence complementary to a target protospacer [8]. This target site recognition process requires a protospacer adjacent motif (PAM), which is NGG in the case of the widely used Cas9 derived from *Streptococcus pyogenes* [9]. Upon the Watson-Crick base pairing of guide RNA (gRNA) and target sequence, Cas9 cuts both complementary and non-complementary strands to create a DSB (**Figure 1**). NHEJ or HDR can repair the cut DNA, with the

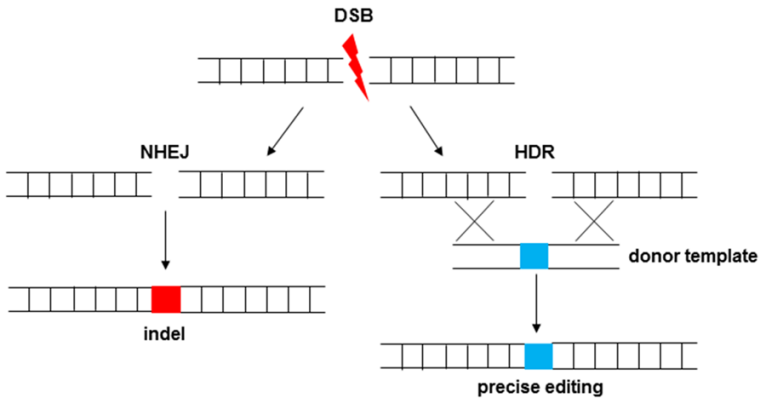

Figure 2. Cas9-initiated genomic editing. After Cas9 cuts both DNA strands, the genomic sequence is repaired by NHEJ or HDR, with the latter one being able to edit DNA sequences precisely in the presence of donor DNA. DSB: double-strand break; NHEJ: non-homologous end joining; HDR: homology-directed repair; indel: insertion and deletion.

latter one being able to correct mutated sequences precisely in the presence of donor DNA (**Figure 2**). CRISPR/Cas9, with its simplicity and multiplexability, greatly facilitates efficient genome engineering, and has led to pioneering development in the whole biological realm [10].

Apart from the current CRISPR/Cas9 system developed from the bacterial type II CRISPR/Cas adaptive immune system in *Streptococcus pyogenes*, many other systems of CRISPR are also being widely explored. Cpf1 is a class 2, type V CRISPR system with a two-component RNA-programmable DNA nuclease. It triggers a staggered DNA DSB with a 4 or 5-nt 5' overhang [11]. CRISPR/C2c2, a system developed from the type VI CRISPR/Cas adaptive immune system in *L. shahii*, is another groundbreaking tool to help scientists target the RNA profile [12]. As the CRISPR/Cas9 system is more mature than others and has been extensively used in the field of gene therapy, this review will focus on the recent advances of this system in the fight against hematological disorders.

Correction of disease-associated mutations in hematological diseases using CRISPR/Cas9

Sickle cell disease

Hemoglobin A is the main type of hemoglobin in human adults, which consists of two alpha and two beta chains. Sickle cell disease (SCD) is an autosomal recessive genetic disorder caused by a single nucleotide mutation (A to T), and the glutamate-to-valine substitution at the sixth residue of the β-globin gene (HBB) leads to the polymerization of hemoglobin and makes a sickle shape of red blood cells. As one of the molecular diseases with clearly elucidated molecular basis, SCD is a hotspot in the field of gene therapy. Great efforts have been made to correct the mutant *HBB* gene in human iPSCs using ZFNs and TALENs [13, 14].

The CRISPR/Cas9 system was used to target this pathogenic missense mutation in SCD patient-derived iPSCs. With a donor DNA template, researchers successfully corrected one mutated allele in these iPSCs, which were then differentiated into erythrocytes. About 6%-10% of the differentiated cells exhibited characteristics of reticulocytes. Moreover, 16-kD β-globin expression was observed in these erythrocytes [15]. Similarly, iPSCs derived from human SCD patient keratinocytes were correctly edited using CRISPR/Cas9 [16]. Patient-derived CD34+ hematopoietic stem and progenitor cells (HSPCs) were another option for gene therapy. CRISPR/Cas9 could induce over 18% gene modification in CD34+ cells in vitro. Cas9 mRNA and an integrase-defective lentiviral vector (IDLV) carrying a gRNA and a correct β-globin gene template were delivered into SCD-derived bone marrow CD34+ cells, and these cells restored the ability to produce wild-type hemoglobin A [17]. More recently, researchers corrected patient-derived HSPCs using Cas9 ribonucleoproteins (RNP) and a homologous donor incorporated in an adeno-associated viral vector. These cells could be differentiated into erythrocytes and express hemoglobin A messenger RNA [18]. Another team took a step further to transplant ex vivo treated human HSPCs into immunocompromised mice and found that these cells maintained SCD gene edits throughout 16 weeks [19]. These preclinical studies prove that gene edited iPSCs and HSPCs retain the ability to differentiate into mature progeny, which represents a research trend towards CRISPR-based

cell therapies for treating hematological genetic disorders.

β-thalassemia anemia

β-thalassemia anemia consists of a group of inherited blood disorders with great genetic heterogeneity including genomic substitution, deletion or insertion that affect the β-globin gene and lead to reduced or absent synthesis of β-globin. In 2014, scientists generated iPSCs from a patient doubly heterozygous for two β-thalassemia mutations (-28 (A/G) mutation of the promoter and the 4-bp (TCTT) deletion at codon 41 and 42 of exon 2). They corrected *HBB* mutations with the combination of CRISPR/Cas9 and a *piggyBac* transposon. The corrected iPSCs retained full pluripotency, exhibited normal karyotypes, and could produce HBB when differentiated into erythroblasts. There was no clone with both mutations corrected, but this is still impressive because clinical manifestations can be alleviated even in the heterozygous state [20]. Similarly, CRISPR/Cas9 and *piggyBac* transposon donor were used in another study to target the intron 2 mutation site IVS2-654 in the globin gene. Both TALENs and CRISPR/Cas9 corrected this mutation in β-thalassemia patient-derived iPSCs and these cells retained pluripotency, but TALENs displayed higher homologous gene targeting efficiency and less off-target events. Therefore, only TALENs-targeted clones were selected for further hematopoietic differentiation analysis, and the derived erythroid cells displayed an increased expression of *HBB* gene compared to those obtained from parental iPSCs [21]. These results suggest that the combination of CRISPR/Cas9 and the *piggyBac* system serves as a feasible gene targeting strategy to achieve gene correction without leaving any footprint. In addition, *HBB* gene CD41/42 (-CTTT) mutation in β-thalassemia iPSCs were corrected by the combination of CRISPR/Cas9 and single-strand oligodeoxynucleotides (ssODNs). Purified corrected clones were also proved to retain full pluripotency and normal karyotype. Corrected iPSCs were further differentiated and the expression of HBB protein was restored. It is also notable that whole-exome sequencing detected no high mutation load in the exomes of the corrected iPSCs, which is an essential requirement for translating this technique into clinical practice [22]. More recently, CRISPR/Cas9 was adopted to correct the homozygous 41/42 deletion of *HBB* gene in patient-derived iPSCs followed by differentiation into HSCs. These HSCs were transplanted into NOD-scid-IL2Rg-/- (NSI) mice. They successfully survived, differentiated, and produced HBB in *in vivo*. No tumor formation was observed in NSI mice [23]. This *in vivo* study indicates that the combination of CRISPR/Cas9 and iPSC is a promising method to correct disease-causing mutations while transplantation of HSCs derived from corrected iPSC can make this feasible in clinic.

One challenge of this strategy is to achieve globin-switch to adult type, because erythroid precursors generated from iPSCs and embryonic stem cells (ESCs) preferentially express embryonic (Є) and fetal (γ) globin rather than adult hemoglobin protein [24, 25]. In contrast, reactivation of fetal hemoglobin production serves as another experimental approach to treat β-thalassemia anemia. Clinical observations indicate that mutations like deletions of the β-globin gene cluster are more likely to respond to this therapy. A pair of upstream and downstream breakpoint gRNAs were delivered with Cas9 to delete 13 kb of the β-globin locus in normal hematopoietic stem and progenitor cells. The erythroid colonies differentiated from these cells exhibited higher γ-globin levels [26]. As initial efforts to augment γ-globin expression with cytotoxic drugs failed to achieve satisfactory results, this strategy of modifying the genome may open a new avenue for treating β-thalassemia anemia by reactivation of γ-globin.

Hemophilia A

Hemophilia A is a bleeding disorder caused by mutations in the *F8* gene, which is located on the long arm of X-chromosome and encodes the coagulation factor VIII. Chromosomal inversions involving introns 1 and 22 of the *F8* gene represent about 25% of all hemophilia A cases [27]. A Korean group generated iPSCs from the urine sample of one patient with intron 1 inversion and another two patients with intron 22 inversion. They chose unique target sequences and adopted gRNAs with two extra, unmatched guanine nucleotides at the 5' terminus to improve targeting specificity. The two large inversions were successfully reverted in hemo-

philia iPSCs, and these cells were subsequently picked out and differentiated into endothelial cells. These endothelial cells not only expressed correctly spliced factor VIII but also rescued factor VIII deficiency after being transplanted into hemophilia A mice. Targeted deep sequencing and whole-genome sequencing analyses identified no off-target mutations in corrected single-cell derived clones [28]. Their results indicate that genetic correction of chromosomal inversions in hemophilia A patient-derived iPSCs using CRISPR/Cas9 could be a potential therapy in the future. Actually, chromosomal rearrangement is a common feature in a number of hematological diseases, such as myelodysplasia syndrome [29], leukemia [30, 31], and lymphoma [32]. The successful correction of two recurrent, large chromosomal inversions in iPSCs derived from hemophilia A patients inspires researchers to explore the application of CRISPR/Cas9 in a wider range of hematological diseases with chromosomal inversions or other large rearrangements.

Hemophilia B

Hemophilia B is an X-linked recessive disease with a deficiency of coagulator factor IX and an increased propensity for hemorrhage. Over a thousand kinds of mutations in human *F9* gene have been defined [33]. Y371D is newly defined *F9* mutation in a family with hemophilia B. Mice carrying this mutation displayed a bleeding phenotype. Two different therapeutic combinations were used to correct the mutation in adult mice: naked DNA constructs (a 120-nt ssODN or a plasmid) and adenoviral vectors. The indel and HDR rate were both 0.56% in the ssODN group. A 4.39% modification rate were obtained in the plasmid donor group, including 2.84% indel mutations and 1.55% G > T corrections. Actually, even a 0.56% correction rate in hepatocytes was sufficient to restore hemostasis in mouse models. On the other hand, the high-dose adenoviral delivery system triggered a higher editing efficiency of 31.34% (NHEJ: 25.81% and HDR: 5.53%), but no therapeutic effects were detected due to severe hepatic toxicity of the recombinant adenovirus [4]. This is a good example of using CRISPR/Cas9 to establish mouse models to facilitate medical research. It also suggests that a safe and efficient delivery system is of great importance in CRISPR/Cas9-mediated gene therapy. The plasmid donor achieved higher efficiency than ssODNs in this study, in contrast to a previous study, which indicates that further research is needed [34]. Hydrodynamic injection was used to deliver both DNA vectors this time, but it is impractical for clinical use at least under current conditions. Non-integrating adenoviral mediated gene delivery achieved the highest gene modification rate in this study but the serious immunogenic side effects still remain a challenge.

Leukemia

ASXL1 is an epigenetic modifier and tumor suppressor in chronic myeloid leukemia (CML), and its mutations are associated with poor clinical outcome [35, 36]. A mutant CML cell line KBM5 harboring an *ASXL1* homozygous nonsense mutation (c.2128G > T, p.G710X) displayed a lack of ASXL1 protein expression. In the presence of a 140-nt ssODN containing the wild-type G nucleotide, this mutation was corrected by the CRISPR/Cas9 system. The corrected cells re-expressed functional ASXL1 protein, and exhibited reduced cell growth and increased myeloid differentiation. Moreover, mice xenografted with corrected KBM5 cells exhibited significantly longer survival than those with uncorrected xenografts [37]. This is an exceptional example of utilizing CRISPR/Cas9 to correct an acquired mutation involved in leukemia. In recent years, great efforts have been made to discover a wider range of cancer driver genes, even in a patient-specific manner. Targeting driver genes is likely to yield twice the result with half the effort in cancer therapy. With its simplicity and multiplexability, CRISPR/Cas9 can be used to correct acquired driver mutations or target several genes simultaneously, which holds promise for achieving personalized cancer therapy. On the other hand, hematological diseases such as paroxysmal nocturnal hemoglobinuria also harbor acquired mutations and can be the next candidates for gene therapy using CRISPR/Cas9.

Fanconi anemia

Fanconi anemia is a genetic disease caused by mutations to a group of genes in the complex Fanconi anemia pathway which repairs DNA inter-strand crosslinks, and patients are predisposed to bone marrow failure as well as tumor formation [38]. HSCT can cure the bone mar-

Table 2. Combating off-target effects

		Strategy	Reference
Predicting off-target effects		CRISPR Design Tool	[3]
		CRISPOR	[44]
		GUIDE-seq method	[63]
		PhytoCRISP-Ex	[64]
		Cas-OFFinder	[65]
		Off-Spotter	[66]
		E-CRISP	[67]
Reducing off-target effects	Single guide RNA	Truncated single guide RNA	[47]
		5' terminus guanine	[28]
	Cas 9	Double-nicking	[48]
		Dimeric CRISPR RNA-guided FokI nucleases	[68]
		Inducible assembly of split Cas9	[69]
		Cas9 PAM variants	[70, 71]
		Programmable DNA-binding domain (pDBD)	[50]
		Cas9 mutants	[72, 73]

row manifestations of Fanconi anemia, but it is not helpful for the physical abnormalities and has the defects of a lack of HLA-matched donors and severe GVHD, which are mentioned above. Therefore, gene therapy is an urgently needed alternative for the treatment of this disease. Previous efforts were made to target the *FANCA* gene into the AAVS1 safe harbor locus in fibroblasts from Fanconi anemia patients using ZFNs and IDLV. EGFP analyses revealed that around 40% of Fanconi anemia patient-derived fibroblasts were edited without any selectable drug after 42 days in culture. Gene-edited fibroblasts were reprogrammed into iPSCs and then re-differentiated into hematopoietic progenitors characterized by a disease-free phenotype [39].

Similarly, fibroblasts derived from a patient with Fanconi anemia was used as a model in another study which adopted the CRISPR/Cas9 system [40]. In addition to traditional Cas9 nuclease, D10A Cas9 nickase was also employed to target the *FANCC* gene. With a D10A mutation in RuvC, one of the two conserved nuclease domains of Cas9, this nickase triggers a break on one single DNA strand rather than generating a DSB [10]. The results showed that both systems could correct the disease-causing mutation, restore proper mRNA splicing and functionally rescue the Fanconi anemia phenotype. The efficiency of Cas9 nuclease and nickase in correcting the *FANCC* c.456+4A > T mutation was compared, and Cas9 nickase achieved higher correction efficiency. Cas9 nickase preferentially mediated HDR and minimized NHEJ in this study, which is a good lesson for researchers who are interested in repairing genes precisely in the future [40]. This same group then took a step further and edited iPSCs derived from a *FANCI* individual harboring the c.1461 T > A nonsense mutation and c.3058+4A > G intron mutation. Fibroblasts from this patient were used to generate iPSCs by the non-integrating Sendai virus reprogramming methodology [41]. Then, the c.1461T > A mutation in exon 15 of *FANCI* was successfully corrected using the combination of CRISPR/Cas9 and a template [42]. Moreover, *in vitro* directed differentiation of the edited cells generated CD34+CD38- cells, which were capable of engrafting and displayed consistent phenotype with cord blood-derived cells [42, 43]. Fanconi anemia is one kind of aplastic anemia, and patients usually have depleted numbers of HSPCs. Therefore, iPSCs are a more promising option for genomic editing than patient-derived HSCPs. This study shows that it is feasible to differentiate corrected iPSCs into cells with characteristics of HSPCs, which holds potential for the personalized autologous therapy of Fanconi anemia.

Current challenges

Combating off-target effects

To date, a great number of online tools have been developed to predict CRISPR off-target

effects (**Table 2**). CRISPR Design Tool (http://tools.genome-engineering.org) integrates the mismatch position, mismatch quantity, guide sequence and transfected concentration of Cas9 and sgRNA to predict likely off-target sites in the target genome [3]. CRISPOR (http://crispor.org), is another gRNA selection tool developed later which integrates both off-target and on-target scoring algorithms to help select efficient gRNA sequences for more than 120 genomes [44]. In silico models which take cell-type heterogeneity into consideration are built to assess the current sgRNA design tools. With the help of bioinformatics and computational techniques, personalized sgRNA design and genome editing with less off-target effects are on the way [45].

In addition to predicting off-target locus, a variety of methods have also been developed to reduce off-target effects. In general, these strategies fall into two categories: gRNA optimization and Cas9 improvement. Both sgRNA length and composition are associated with precise gene targeting. Various computational approaches have been developed to assist the design of optimal sgRNAs, and this have been reviewed [46]. As for sgRNA length, truncated gRNAs of 17 or 18-nt can decrease off-target effects induced by individual Cas9 nuclease or paired Cas9 nickases. Specifically, undesired mutagenesis at some off-target sites can be reduced by 5,000-fold or more with on-target genome editing remained similar to their full-length counterparts [47]. On the other hand, Cas 9 has been modified into Cas9 nickases, split into two parts to form inducible assembly, or fused with programmable DNA-binding domain (pDBD) or FokI nucleases. Cas9 contains two catalytic residues, HNH and RuvC, which cleave the DNA on both strands to form DSBs [8]. D10A mutation in RuvC renders Cas9 only able to trigger single-strand nicks. When this D10A mutant nickase is paired with offset guide RNAs, the off-target activity can be decreased by 50- to 1,500-fold [48]. Cas9 nickase mRNA and sgRNA were co-injected into one-cell mouse embryos in a study, results revealed that no mutagenesis were detected at predicted off-target sites [49]. Cas9-pDBD chimera made by fusing a programmable DNA-binding domain to Cas9 can not only improve precise gene manipulation but also increase the targeting range so greatly that this system can target nearly any genomic locus [50]. In addition, Cas9 in the form of RNP can also reduce off-target, which will be discussed in the next part [51].

Safe and efficient delivery

The CRISPR/Cas9 system has to be delivered to the nuclei of targeted cells to edit the genome. To apply CRISPR/Cas9 to human beings in the future, a safe and efficient delivery system is a precondition. Listed in **Table 3** are various methods adopted in previous studies to deliver the CRISPR/Cas9 system (**Table 3**). An optimal way to deliver the system needs to be efficient and safe. Early efforts tried to encode Cas9 protein together with gRNA in a plasmid, which is simple and displays low immunogenicity [3]. A great number of studies have used this approach to edit cellular genome by chemical transfection or electroporation. However, chemical transfection is only efficient in certain cell types, such as the HEK293 cell line. For cell lines that are difficult to transfect by chemical reagents, electroporation can be helpful at the expense of a higher cell death rate.

Apart from those two established methods, viruses are a more controversial approach. Viruses are used in gene therapy due to their characteristics of a larger capacity and a higher transfection rate. However, their applications are greatly flawed because of the random insertion of virus into the genome, which arouses a serious safety issue. A teenager treated with a very high dose of the therapeutic adenoviral vector died from severe immune complications in a liver gene therapy trial for ornithine transcarbamylase deficiency [52]. In another study to treat X-linked severe combined immunodeficiency, the retroviral vector was integrated in proximity to the *LMO2* proto-oncogene promoter, leading to transcription and expression of *LMO2* and subsequent leukemia [53]. Immunogenicity is another serious problem. In the study aimed to correct the *F9* gene mutation, ssODN/plasmid-based delivery systems were compared with recombinant adenovirus. Although recombinant adenovirus achieved a higher HDR and NHEJ rate, the bleeding phenotype was not rescued due to its severe immune response-mediated hepatic toxicity [4]. Adeno-associated virus (AAV) delivery vehicle, which

Table 3. CRISPR/Cas9 delivery methods

Cas9 type	Delivery form	Delivery method	Cell line (model)	Reference
DNA constructs	Plasmid	Chemical transfection	Induced pluripotent stem cells (iPSCs)	[22]
	Plasmid	Electroporation	Human CD34+ cells	[17]
	Adeno-associated virus (AAV)	Tail vein injection	Mouse	[74]
	Recombinant adenovirus (Adv)	Tail vein injection	Mouse	[4]
	Plasmid	Microfluidic membrane deformation	Hard-to-transfect cells	[75]
mRNA constructs	mRNA	Microinjection	Monkey embryo	[76]
		Electroporation	HEK293FT cell, U2OS cell, Human iPSCs et al	[77]
	Cas9 mRNA with AAV encoding a sgRNA and a repair template	Lipid nanoparticle-mediated delivery of Cas9 mRNA	Mouse	[78]
	Lentiviral vector	Transduction	HEK293T cells	[79]
Cas9 protein	Ribonucleoproteins (RNP)	Cationic lipid-mediated delivery	Mouse cochlea	[80]
	RNP	Electroporation	Human T cell	[81]
	DNA nanoclew	Incubation and intra-tumoral injection	U2OS.EGFP cell and U2OS.EGFP tumor-bearing mouse	[82]
	Cell-penetrating peptide-conjugated recombinant Cas9 and CPP-complexed guide RNA	Cell-penetrating peptide-mediated delivery	Embryonic stem cell, dermal fibroblast, HEK293T cell, HeLa cell, and embryonic carcinoma cell	[83]
	Recombinant Cas9 protein and *in-vitro*-transcribed sgRNA	iTOP system	KBM7 cells and human embryonic stem cells	[84]

has the properties of broader range of serotype specificity, low immunogenicity and non-integration, is an alternative in genetic research [54, 55]. However, due to its low packaging capacity (~4.5 kb), the smaller Cas9 orthologues from *Staphylococcus aureus* (SaCas9) might be a better choice to carry out gene editing [54]. Therefore, to translate this method into an established clinical therapy, the random insertion and immunogenicity problems must be tackled beforehand.

In addition to DNA constructs, Cas9 in the form of mRNA and ribonucleoprotein have also been explored. Cas9 mRNA delivery is less likely to trigger uncontrolled integration of unwanted sequence into the host genome compared to the plasmid method. Its defect is that the stability of mRNA is not as good as DNA. As for the ribonucleoproteins consisting of Cas9 protein and gRNA, less off-target effects are recorded due to the easy degradation of Cas9 protein upon administration [51].

Future perspectives

The emergence of CRISPR has triggered a revolution in the biotechnology world and ignited an explosion of interest in gene editing to eradicate genetic diseases. Its easy operation, low cost, short work cycle and multiplexing functions are unparalleled and make it popular throughout the biomedical society. Gene therapy developments are emerging in an endless stream since the introduction of CRISPR.

Hematological system is one of the first systems to witness the exciting breakthroughs in the field of gene therapy. Scientists have taken advantages of this technology to construct genome-wide mutation libraries for screening of disease-causing genes, generate useful cell lines and genetically engineered animals for disease modeling, and carry out gene corrections both *in vitro* and *in vivo*. CRISPR has opened up a new avenue for treating hematological disorders.

To treat hematological disorders, a clinically relevant cell type is of great importance. A decade ago, Yamanaka and Takahashi published that mouse embryonic cells and adult fibroblasts could be reprogrammed into embryonic-like iPSCs by introducing four genes: *Oct3/4, Sox2, Klf4 and c-Myc* [56]. Unfortunately, this pioneering technology is still restricted to the field of biological research, mainly due to an unsuccessful clinical trial to treat age-related macular degeneration in which two small genetic changes in both patients' iPSCs and the iPSC-derived retinal pigment epithelium were identified [57]. However, with the emergence of CRISPR technology, iPSCs are recently revitalized and widely used to investigate therapeutic strategies for hematological disorders. It is a promising method to modify patient-derived iPSCs by CRISPR *in vitro* and re-implant them back. This personalized therapy provides abundant cell resources for transplantation, eliminating the cumbersome process of finding an HLA-matched donor in the traditional transplantation way to treat hematological diseases. Due to the specificity of hematological system, the easily accessibility of HSCs provides another feasible option to do gene therapy. Autologous transplantation is a routine technique in diseases like lymphoma and myeloma. Therefore, when the gene editing issue is solved, this method is very likely to be firstly introduced into clinical practice. Another noteworthy technology is chimeric antigen receptor T-cell immunotherapy (CAR-T). The exciting therapeutic effects of CAR-T in chronic and acute lymphoid leukemia reveal its magic in treating cancers [58, 59]. Further advances involving the combination of CAR-T and CRISPR should lead to great breakthroughs within the next decade.

On the other hand, there is still a long way to go before making this new therapeutic option available in clinic. Of the problems to be tackled, combating off-target effects and finding an efficient and safe delivery system are of great concern. In addition to the techniques listed in this review, tremendous efforts on other aspects have been made. For example, the smaller Cas9 orthologue SaCas9 is capable to edit the genome with efficiencies similar to SpCas9 [54]. The extreme structural and functional diversity of CRISPR/Cas systems are well reviewed elsewhere, and we could harness the potential of different CRISPR/Cas systems for different purposes with great flexibility in future biotechnology [60]. Another challenge is to combat the annoying off-target effects. Great progress has been made in predicting the off-target sites, and various strategies concerning the improvement of sgRNA and Cas9 have

been established. Numerous online websites are making the design of sgRNA a more personalized choice. Modifications of the components of the gene targeting system also greatly improve the specificity.

The Chinese You Lu's team has launched a clinical trial to treat non-small cell lung cancer by shutting down the cell surface receptor *PD-1* gene in patient-derived T lymphocytes using CRISPR/Cas9 in 2016 [61]. Another team led by Carl June will combine CAR-T and CRISPR to treat myeloma, melanoma and sarcoma [62]. Hopefully, CRISPR/Cas-mediated in situ genome editing will become a feasible therapeutic strategy for human hereditary diseases in the near future.

Acknowledgements

This study was funded by Jiangsu Provincial Special Program of Medical Science (BL2012005); Jiangsu Province's Key Medical Center (ZX201102); The Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD); National Natural Science Foundation of China (81402176); Natural Science Foundation of Jiangsu Province (BK20140288); Natural Science Foundation of Jiangsu Higher Education Institutions of China (14KJB320011).

Disclosure of conflict of interest

None.

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