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Review

CRISPR/Cas9 system and its applications in human hematopoietic cells

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ABSTRACT

Since 2012, the CRISPR-Cas9 system has been quickly and successfully tested in a broad range of organisms and cells including hematopoietic cells. The application of CRISPR-Cas9 in human hematopoietic cells mainly involves the genes responsible for HIV infection, β-thalassemia and sickle cell disease (SCD). The successful disruption of *CCR5* and *CXCR4* genes in T cells by CRISPR-Cas9 promotes the prospect of the technology in the functional cure of HIV. More recently, eliminating *CCR5* and *CXCR4* in induced pluripotent stem cells (iPSCs) derived from patients and targeting the HIV genome have been successfully carried out in several laboratories. The outcome from these approaches bring us closer to the goal of eradicating HIV infection. For hemoglobinopathies the ability to produce iPSC-derived from patients with the correction of hemoglobin (*HBB*) mutations by CRISPR-Cas9 has been tested in a number of laboratories. These corrected iPSCs also show the potential to differentiate into mature erythrocytes expressing high-level and normal HBB. In light of the initial success of CRESPR-Cas9 in target mutated gene(s) in the iPSCs, a combination of genomic editing and autogenetic stem cell transplantation would be the best strategy for root treatment of the diseases, which could replace traditional allogeneic stem cell transplantation.

Contents

1. Introduction

Genome-editing technologies have emerged as powerful tools to manipulate gene expression for basic research and gene therapy. ZFNs (Zinc-finger nucleases) [93], TALENs (Transcription activator-like effector nucleases) [64] and CRISPR-Cas (clustered regularly interspaced short palindromic repeats-crispr-associated genes) are three most commonly used tools in gene editing over the last 10 years. Among these three, CRISPR-Cas, more specifically, CRISPR-Cas9 (Cas9 = crispr-associated protein 9 nuclease) technology is the latest player in the field [49]. ZFNs are the first and most established genome-editing tool. The ZFNs have two domains: zinc finger DNA-binding domain and DNA-cleavage domain. The DNA-binding domain (transcription factors) recognizes and binds specific target DNA sequence (each finger module recognizes 3–4 nucleotides). The binding enables the cleavage domain to break the double-strand DNA, which triggers DNA repair process, either by "non-homologous end joining" or "homology-direct

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repair". Typically, the DNA-cleavage domain is the restriction enzyme FokI [54,93]. The DNA binding domain in TALENs is TAL effectors built from arrays of 33 to 35 amino acid modules. Each module recognizes a single nucleotide. With different combinations of the modules investigators can target nearly any genes in a wide range of cell types and organisms. The TAL effector proteins are derived from the bacteria genus *Xanthomonas*. Similar to ZNFs, TALENs also use FokI to cut target DNA [64]. Since TALENs are much larger molecules than ZFNs, it may affect the efficiency of the delivery of molecules into cells. CRISPR-Cas is a prokaryotic remarkable immune system that is used by many prokaryotes to detect and eliminate genetic intruders ([89,96,101]). Since its pioneers, Jennifer Doudna and Emma Nuelle Charpentier, published the first paper describing the application of CRISPR-Cas9 system in editing genomic DNA in 2012 [49], thousands of CRISPR-Cas9 papers have been published. This modified system has turned out to be a revolutionary biotechnology. Today CRISPR-Cas9 is considered the most efficient and advanced tool to target and modify genome DNA due to its simplicity, efficiency and versatility [22,27,28,34,35,46,50,56,99]. CRISPR-Cas9 was selected as "the breakthrough of the Year" (2015) by Science. In this short article I will briefly review the discovery of CRISPR-Cas9 system and then focus on applications of CRISPR-Cas9 in genome editing in human hematopoietic cells. For comparison and understanding of related research data, the applications of other two genome editing technologies, ZFNs and TALENs will also be described briefly.

2. Brief history of the CRISPR SYSTEM

In the late 1980s, researchers observed unique short palindromic repeat sequences in *E. coli* genomes [47,77], however, their function was not established at that time. Later, the similar repeats were found in some other bacteria and most archaea [11,73–75]. Based on current study, about 40% of sequenced bacteria and ~90% of sequenced archaea genomes have CRISPR loci [32,33,55]. In general, the repeats are 20 to 50 bp long and vary with species. Subsequently, a group of genes was found to be associated with CRISPR repeats, which was named CRISPR-associated genes, or *Cas*. As of today, the cluster of *Cas* family has as many as 45 members [36]. The Cas genes are often adjacent to the repeat genes and encode a number of nucleases or helicases which are able to cut or unwind DNA [48]. The repeats were separated by distinct short DNA sequences called "spacers". It was noted that the spacers have similar base pairs as the repeats in length but the sequences for spacers are not identical [32,33,36,55,68,69]. Surprisingly to investigators, these peculiar spacers were found to be part of phage DNA and extra chromosomal plasmid DNA [9,30,76,81]. These findings suggest that the spacers were taken by bacterial/archaea sometimes during their previous life. Further studies showed that the species containing a phage DNA fragment are resistant to corresponding phage infection, whereas the species without "spacers" are sensitive to phage invasion. Thus, CRISPR-Cas turns out to be an immune system of prokaryotes [39,68,69,72,80].

According to current classifications CRISPR-Cas systems could be grouped into two classes: class I and class II. The class I systems use a complex of multiple Cas proteins to disrupt foreign nucleic acids; the class II systems only employ a single large Cas nuclease for the same purpose. Each of the classes has several types and each type can be further divided into multiple subtypes with a total of 16 subtypes identified [67].

The CRISPR-Cas immune process carried by prokaryotes consists of three major steps: spacer acquisition, CRISPR transcription, and interference. When a CRISPR-positive prokaryote is attacked by a virus, the DNA fragment of the virus is taken up by the prokaryote and stored in the CRISPR locus as a spacer(s) that as a form that memorizes the foreign DNA sequence of the host defense against subsequent invasions of the corresponding intruders (virus or plasmid). This immune process requires Cas enzyme(s) [1,37,90,106]. During the transcription process, CRISPR is transcribed to CRISPR RNA (crRNA). The primary transcript is the precursor of crRNA with long base pairs (pre-crRNA) that is then cleaved to a mature crRNA [10,13,18,58,59]. In the class II system, the transcription process requires additional formation of a small RNA molecule named trans-activating crRNA (tracrRNA). The tracrRNA is also transcribed from the CRISPR-Cas locus and its base pair sequence is partially complementary to pre-crRNA. After transcription, the tracrRNA binds to the pre-crRNA and promotes formation of mature crRNA [49,85]. At the interference step, the complex of the double strand-RNA (tracrRNA-crRNA) and Cas9 nuclease guides the nuclease to the site-specific DNA sequence of intruders and disrupt the DNA fragment [49,66,83,95,101]. Briefly, when a virus attaches to a bacterium, the bacterium can copy and incorporate the virus DNA into their genome as "spacers" in the locus between the short DNA repeats in CRISPR. These spacers function as a template for CRSPR RNA molecules to recognize and bind to the complementary target DNA sequence(s) when the virus infects the bacterium again. Meanwhile, the RNA molecules guide the effector Cas9 enzyme to and cut the double strand DNA of the invading virus. The simplicity of the class II immune system that only involves three components was first realized by Doudna and Charpentier labs [49], after which they developed a simple two component-CRISPR-Cas9 system, in which tracrRNA and crRNA have been combined into a single guide RNA (gRNA or sgRNA). The gRNA-guided (programmed) Cas9 has been shown to be efficient as the Cas9 programmed by both tracrRNA and crRNA in targeting and cutting target DNA. According to the gRNA-guided CRISPR-Cas9 system, Cas9 is a universal nuclease. As long as the gRNA sequence (about 20 bps) is redesigned based on target DNA specificity, one can apply this technology to any gene locus in a variety of cell types and eventually change the expression of the genes [49].

In early 2013, two groups published the first report of CRISPR-Cas9 applied to human culture cells [16,70] and a single CRISPR-Cas9 array was programmed to edit several target sites at once [16,41,70]. Since 2012 CRISPR-Cas9 has been successfully used in many different cell types from a variety of organisms including human hematopoietic cells in which, the edited genes are linked to various cellular processes and a number of genetic and non-genetic hematopoietic diseases.

3. Disruption of *CCR5*/*CXCR4* and HIV-1 gene

The human immunodeficiency virus (HIV) is one type of retrovirus, which causes human acquired immunodeficiency syndrome (AIDS). Today, AIDS is considered an incurable disease, although the progress in anti-retroviral therapy has reduced significantly the mortality rate of AIDS patients and increased patients' life span. The primary target of HIV-1 in human is CD4+ T cells (Helper T cells). During infection, the gp120 envelope glycoprotein of the viruses binds to CD4 proteins on the surface of the CD4+ T cells followed by injection of the virus nucleic acids. Further studies showed that the binding of CD4 is not sufficient for HIV entering the T cells. Scientists have found that two chemokine receptors, CCR5 [2,21,23] and CXCR4 [25], are also required for many types of HIV infections. Thus, these two molecules become the co-receptors of HIV for entering the CD4+ T cells. Later, researchers realized that individuals carrying a CCR5 mutation known as CCR5-Δ32 (32 bp deletion on both alleles of the gene) are resistant to HIV infection [20]. HIV-1 infection often leads to the establishment of a state of latent reservoir in which HIV integrates its genetic materials into the genome of host cells as proviral DNA that replicates along with the host without producing active HIV. Only when latently infected cells are reactivated, these proviral DNA start transcription followed by translation and finally produce new HIV [51]. Although T cell genome engineering appears a straightforward manner to treat HIV infection, fundamentally, there are two strategies for HIV scientists and physicians to consider in HIV research and clinical trial: a functional cure and an eradicating cure [97]. The functional cure is able to eliminate the active virus existing in HIV patient blood; the eradicating cure attempts to remove completely all viruses from the patient's body, which not only removes the virus from the blood but also eliminates those in the latent reservoir.

For a functional cure, current research focuses on CCR5 and CXC4R impairment. Both molecules are co-receptors of HIV but CCR5 is a major one. Successful disruption of the *CCR5* gene has been reported from several labs [3,45,91,105]. ZFNs were the first gene editing technology applied to disrupt *CCR5* gene in patient-derived CD4+ T cells [91]. In this study, 12 HIV patients were enrolled and received autologous CD4+ T cells that were edited by the ZFNs. After 1 week, the authors found that the average number of CD4+ T cells significantly increased and the blood level of HIV DNA decreased in most patients. Their data suggest that *CCR5* targeting by ZFNs protects CD4 T cells and gains partial success in obtaining resistance to HIV infection. Since 2013, CRISPR-Cas9 has also been extensively tested for its ability to edit/disrupt *CCR5* [17,100]. It has been reported that the lentiviral vectors expressing Cas9 and gRNAs introduced into human CD4+ T cells yielded high frequencies of *CCR5* gene disruption and the cells expressing mutated CCR5 were resistant to R5-tropic HIV-1 infection [100]. The off-target is not a big concern in this study, because by using T7 endonuclease I assay, the authors could not detect genome mutations at potential off-target sites for 84 days after transduction [100]. Since T7 endonuclease is not very sensitive to screen mutations, the other methods may be needed to obtain more reliable off-target data. Similarly, the disruption of *CXCR4* in T cell lines and primary T cells by CRISPR-Cas9 also led to resistance of the cells to HIV infection [40,86]. Schumann et al. [86] used Cas9: sgRNA ribonucleoproteins (Cas9 RNPs) to ablate *CXCR4* in T cells by random insertion and deletion. With this method, they obtained about 20% targeting efficiency with decreased levels of CXCR4 in up to ~40% of the cells being observed [86].

Although disruption of *CCR5* or *CXCR4* in mature T cells is a successful approach to block or decrease HIV entry, it is difficult to remove all *CCR5* or *CXCR4* genes in all CD4+ T cells such as those found in the peripheral blood, spleen, lymph nodes, and intestine. The other limitation of this approach is that it may not be able to ablate the viruses that infect other type of cells such as macrophages. In order to overcome this limitation, several groups have used CRISPR-Cas9 and other gene editing technologies to target *CCR5* gene in induced pluripotent stem cells (iPSCs). The first report of iPSCs with *CCR5-Δ32* was published in July 2014 [105], in which a combination of CRISP-Cas9 or TALENs together with the piggyBac technology was used. The modified gene exactly mimics the natural mutation. After the mutation, those stem cells (iPSCs) were able to differentiate into monocytes/macrophages and were resistant to HIV-1 infection [105]. This work provides another efficient approach toward the functional cure of HIV infection. However, targeting only *CCR5* gene will not provide full protection against viruses using the other co-receptor, CXCR4. The same is true if only *CXCR4* is targeted but not *CCR5*. Furthermore, the latent viruses in infected cells will not be disrupted by this strategy. Thus, as an eradicating cure, disrupting integrated HIV-1 genome in reservoir cells appears critical to remove all viruses from the patient's body.

The first report applying CRISPR-Cas9 to edit HIV gene in *vitro* was published in August 2013 in which the long terminal repeat (LTR) sequence of HIV-1 was cleaved or mutated, resulting in a significant decrease in the virus expression. In addition, the internal viral genes in the host cell chromosome were also removed [24]. Later, a similar successful approach was reported by another group [43]. In this report, a specific target within the HIV-1 LTR U3 region was efficiently edited by Cas9-gRNA, resulting in the inhibition of viral gene expression and replication in latently infected cells. Subsequently, the efficiency of CRISP-Cas9 editing HIV-1 genes was tested in more different cell types. In HIV-1 infected JLat10.6 cells, the engineered CRISPR-Cas9 targeted 10 sites in HIV-1 DNA. As a result, HIV-1 gene expression and virus production were significantly diminished with a 20-fold reduction being observed [109]. Further studies on CRISP-Cas9-mediated CD4+ T cells and -iPSCs provided a good explanation for the high efficiency of CRISPR-Cas9: Cas9 could directly target the viral genomic DNA as well as the proviral elements already integrated in the host genome in human cells [57].

During genome editing for HIV research, CRISPR-Cas9 with single guide RNA is the most common approach used to target the *CCR5* or *CXCR4* gene, however, the efficiency of this approach varies with cell types. For example, the low efficiency of targeting by using single guide RNA has been observed in primary CD4+ T cells [71]. As a result, some researchers constructed a CRISPR-Cas9 vector with two separate gRNAs that recognizes two sites on the same locus. They reasoned that dual gRNA system might generate expectable gene mutations more frequently than single gRNA does. By using this method these researchers indeed observed an increased targeting efficiency in human and mouse cells [52,71,108]. Another way to increase the efficiency with single guide RNA is by using Cas9:sgRNA ribonucleoproteins (Cas9 RNPs). Different from a plasmid-mediated expression of Cas9 and sgRNA (gRNA), in this method, a recombinant Cas9 is mixed with in vitro transcribed sgRNA in a buffer system for a few minutes, after which the complex is transfected into target cells. Recent reports from several labs have demonstrated that Cas9 ribonucleoproteins can increase delivering and targeting efficiency of Cas9 and sgRNA [53,60]. In hematopoietic cells the Cas9 RNPs allowed excision of CXCR4 up to 40% of CD4+ T cells with about 20% targeting efficiency [86] as described above. CRISPR-Cas9 technology can also be used as an activator system that can recruit several transcriptional activation domains to the HIV 5′ long terminal repeat (LTR) within the LTR U3 element, by which the authors were able to activate the silencing HIV provirus and finally eliminate the virus [8].

4. Studies on β-thalassemias and sick cell disease

β-Thalassemia is a group of inherited blood diseases that affect populations in the Mediterranean region, South-East Asia, the Indian subcontinent and the Middle East [15]. This disease is caused by mutations in the β subunit(s) of hemoglobin (HBB) which reduces the production of mature adult β-globin subunits [14]. As a result, the insufficient oxygen carried by the decreased amounts of HBB will not meet the requirement for cellular metabolism. The mutations in the β subunits have many different types such as single base pair change or small fragment deletions. Currently, allogeneic stem cell transplantation is the only way to treat this disease [4,62,79]. However, the extremely low rate to find a full match-donor and the clinical risk from possible immune rejection limit the use of this approach. Thus, generation of induced pluripotent stem cells (iPSCs) from β-thalassemia with corrected *HBB* gene may be the better approach to cure the disease.

Since 2014 a number of laboratories have created iPSCs from different type cells isolated from β-thalassemia patients. The mutated genes were corrected by using CRISPR-Cas9 technology [78,88,102]. In one report, the skin fibroblasts from an anonymous patient who is heterozygous for the −28 (A/G) mutation and for the 4-bp (TCTC) deletion at codons 41 and 42 in exon 2 were reprogrammed in the presence of reprogramming factors. These induced stem cells with mutations were then corrected by CRISPR-Cas9 combined with the piggyback technology. The authors found that the *HBB* mutations in the patient-derived iPSCs were completely corrected and the cells retained full pluripotency. The differentiated erythroblasts from iPSCs expressed normal HBB protein [102]. In another study, the homozygous mutations of CD41/42 (−CTTT) in iPSCs from a β-thalassemia patient was also corrected successfully by a combination of single-strand oligodeoxynucleotides (ssODNs) together with CRISPR-Cas9 technology [78].

Since TALENs have also been used to target *HBB* mutation genes [63,103], one group compared the efficiency in editing *HBB* in patient-derived iPSCs by these two technologies. What they found is that TALENs exhibit higher gene integration efficiency at the specific *HBB* IVS2-654 gene locus with fewer off-target events when compared with the outcome from using CRISPR-Cas9 [103]. More comparison studies from other labs are needed to reach a general conclusion on the efficiency of the process.

One important and interesting research in the area of genomic editing in 2015 was the report of applied CRISPR-Cas9 in human embryo cells [56]. Researchers in Huang's lab employed CRISPR-Cas9 to correct the gene mutations responsible for β-thalassemia in human non-viable embryo cells [56]. In doing so, they injected a complex of gRNA, Cas9 RNA, and ssDNA oligos into tripronuclear zygotes in different concentration combinations. Then, the whole genome was collected and the target region was amplified by PCR followed by DNA sequencing. What they found from this research is that CRISPR/Cas9 could effectively cleave the endogenous β-globin gene (*HBB*) but the efficiency of homologous recombination directed repair (HDR) of *HBB* was low; off-target cleavage was also observed in these 3PN zygotes, suggesting that improvement of the approach is required for further study. This report caused human ethical concern and stirred debate on the topic even though the embryo cells used by Liang were non-viable. Although gene editing technologies have been used widely in human adult somatic cells and animal embryos, this is the first report of CRISPR-Cas9 applied to a human zygote. Many scientists called for full discussions before moving further; some admitted that working on human zygote could eradicate inherited diseases and produce a healthy baby [19].

Sickle cell disease (SCD), also called Sickle cell anemia, is a severe chronic incurable anemia. A single nucleotide change (A to T) in the β gene for the β subunits of hemoglobin causes sickle cell disease. The point mutation in codon 6 of the gene translates the amino acid valine instead of the normal glutamate, leading to production of abnormal hemoglobin (HbS). Polymerization of HbS results in the formation of sickle shape-erythrocytes that are fragile and easily clog the small blood vessels. As a result, the delivery of oxygen to capillaries of body tissues is impaired. As with β-thalassemia, today, the only available cure for SCD is allogeneic stem cell transplantation. As described above the wide range application of this approach is hampered by extremely low match rates between a donor and recipient and possible risk of graft rejection. The current advances in genome editing make autologous stem cell transplantation available to SCD patients in near future.

ZFN was the first gene editing technology used to correct point mutations in human iPSCs in 2011 [109]. In this report, the iPSCs were generated from the bone marrow stromal cells of SCD patient with 2 mutated β-globin alleles (βs/βs). The approach took two-step corrections (from β^s to β^A), first correcting 1 allele mutation to produce β^s/β^A heterozygous iPSCs and then correcting the second allele mutation to form the second β^s/β^A heterozygous. Due to culture conditions, the differentiated erythrocytes from iPSCs are not fully mature and the expressed HBB of the cells is γ-globin instead of β-globin [109]. It is unclear from the report described above whether non-mature erythrocytes containing nuclei only resulted from the culture conditions or were also affected by other factors.

In another report, three ZFN pairs were used to break double-strand DNA in the iPSCs cells derived from patient's fibroblasts, which increased greatly homologous recombination frequency. The successful gene corrections were then examined by PCR, Southern blots, and DNA sequence [87]. By using ZFNs designed to flank the sickle mutation and co-delivering a homologous donor template, Hoban et al. showed, in 2015, high levels of gene targeting and modifications in $CD34^+$ hematopoietic stem and progenitor cells derived from the SCD patients. Furthermore, the modified cells maintained their multilineage differentiation ability, leading to the formation of myeloid and erythroid clones and produced wild-type hemoglobin tetramers [38]. Together, the outcome from using ZFNs supports the continued development and application of the gene editing technology in SCD research and clinical trials.

As described above the CRISPR-Cas9 system is the latest advanced genome editing technology, which has also been applied to SCD research. At least in one report, CRISPR-Cas9 has been reported to be more efficient than ZFNs and TALENs in targeting *HBB* locus near the SCD point mutation in human iPSCs [44]. In this study, they used Cas9 and gRNA that target *HBB* 20-bp downstream to the β^s mutation. Meanwhile, a donor vector containing wild-type *HBB* DNA was used to correct the β^s mutation. The authors found that they readily corrected one allele of the SCD *HBB* gene in human iPSCs. Meanwhile, they also detected the 16-kD β-globin protein expressed from the corrected *HBB* allele in the erythrocytes differentiated from genome-edited iPSCs. The expression levels of HBB protein are similar to that of erythrocytes differentiated from untargeted iPSCs [44].

Hereditary persistence of fetal hemoglobin (HPFH) is a benign condition in which significant fetal hemoglobin (HbF) production has been found in some adult blood. The high levels of HbF can alleviate the clinical course of β-hemoglobinopathies, such as beta thalassemia and SCD [26]. The mutations that cause HPFH often are located on the regions of the extended β-globin gene that regulate expression of HBG1 and HBG2 genes. In order to recapitulate the naturally occurring HPFH-associated mutations for the purpose of alleviating β-hemoglobinopathies, Traxler et al. used a lentiviral vector that expresses mChery (a fluorescent protein), Cas9 and two guide RNAs to delete a 13-nt sequence in the promoters of the HBG1 and HBG2 genes from erythroprogenitor HUDDEP-2 cells that mainly express HbA (adult hemoglobin) and from peripheral blood CD34+ stem/progenitor cells isolated from two healthy adults. What they found, after the transfection of the vectors, is that HbF in the control cells (without 13-nt deletion) remained undetectable; in contrast, the cells transfected with the vector containing Cas9 and gRNAs had a significant increase in HbF levels [92]. Their data suggest another potential DNA target for genome editing-mediated therapy of β-hemoglobinopathies besides the corrections of the mutated gene(s) in β-thalassemia and SCD genomes.

The data described above demonstrate that a single gene mutation can be successfully corrected by gene editing technologies, either ZFNs, TALENs, or CRISPR-Cas9. These gene editing technologies, especially CRISPR-Cas9, provide the prospect for and move a significant step toward gene therapy for hemoglobin disorder patients.

5. Disruption of other genes

5.1. B2M

One of major obstacles for allogeneic stem cell transplantation is its limited application in clinics due to the differences of human leukocyte antigen (HLA) genes: different peoples may have a different type of HLA. The gene *B2M* encodes a protein named beta-2 microglobulin. This protein is associated with the HLA (or major histocompatibility complex-MHC) class I in humans. Deletion of *B2M* in human pluripotent or myeloid stem cells will disrupt the expression of HLA I and produce HLA-negative white cells. The major benefit of *B2M* knockout cells is that they could serve as universal donors [82]. Initial study used the adeno-associated virus (AAV) gene targeting vectors to knockout *B2M* [82]. In 2014, one group tested the efficiency of CRISPR-Cas9-induced deletion of *B2M* in CD4+ T cells and CD34+ hematopoietic stem/progenitor cells (HSPCs). They observed that using single gRNA to delete *B2M* led to low efficient mutagenesis in the T cells (1.4–4.7%) but high efficiency in HSPCs. A dual gRNA approach improved gene deletion efficiency in both cell types [71].

5.2. PUMA

p53 is a tumor suppressor gene. Its protein product, p53, is able to arrest cell cycle, induce apoptosis, and repair damaged DNA. The p53-induced apoptosis is the response to abnormal proliferation signals (for example, cancer) and DNA damage (for example, stress) and involves enormous molecules such as MDM2, PUMA, cytochrome *c*, Apaf-1, and caspase 9. Which of the molecules is key for p53-induced apoptosis is not well established. Nutlin3 is a small chemical molecule that has been used for clinical trials for cancer treatment, because it inhibits the interaction of MDM2 and p53. By using transgenic mouse models and CRISPR-Cas9-mediated targeting *PUMA* (exon 3) in

lymphoma-derived T and B cells, a recent publication shows that PUMA plays a critical role in p53-induced apoptosis in response to Nutlin3 [94].

5.3. BCL11a and CEBPA enhancers

Initiation and regulation of transcription in eukaryotes is a complex process. It involves various components including RNA polymerase, transcription factors, proximal-control elements, as well as DNA enhancers and silencers. An enhancer is a short (50–1500 bp) region of DNA that is located upstream of a transcription start point, within the core-promoter, or downstream of a gene. The main function of the enhancer is to increase the activity of gene transcription. *BCL11a* is a gene encoding the protein named B-cell lymphoma/leukemia 11a in human. This gene is associated with B lymphocyte development, neurodevelopment, and fetal hemoglobin silencing [6,61,84]. The production of abnormal hemoglobin is a common hematopoietic disorder condition. The specific effect of the *BCL11a* enhancer on the expression of hemoglobin has been an interesting subject in recent years. The insight into the organization and function of *BCL11a* enhancer in erythroid progenitor cells were provided in a recent paper, in which 12-kb *BCL11a* enhancer was deleted from HUDEP-2 cells by using CRISP-Cas9 technology [12]. The HUDEP-2 is CD4 + hematopoietic stem and progenitor cell (HSPC)-derived erythroid precursor cell line that expresses BCL11A and predominantly β- rather than γ-globin [12]. After deletion of the enhancer, the expression of γ-globin and fetal hemoglobin (HbF) protein significantly increased. This result suggests that the enhancer sequences could serve as target for therapeutic genome editing for the increase of HbF synthesis in the β-hemoglobin disorder patients [12]. The data also confirmed previous reports that the functions of the enhancer are independent from its orientation in its locus.

CCAAT/enhancer binding proteins (C/EBPs) are a family of factors that regulate cell growth and differentiation. These factors, particularly C/EBPα, play an important role in normal myelopoiesis [29,107]. The C/EBPα is encoded by the *CEBPA* gene in human. By using CRISPR-Cas9 technology combined with other functional genomic approaches Avellino R et al. [5] identified an enhancer located +42 kb from *CEBPA* which is active and engages with the *CEBPA* promoter in myeloid cells only. Deletion of the enhancer in germ line cells from the mice reduced expression of the *CEBPA* gene in hematopoietic stem cells and myeloid progenitors leading to the blockage in myeloid differentiation. They conclude that a single +42 kb enhancer is essential for *CEBPA* expression in myeloid cells only [5].

5.4. SH2B3

SH2B3 encodes an adaptor protein that is involved in the negative regulation of a number of growth factor- and cytokine-induced signaling pathways. These signal pathways are critical in the development and function of hematopoietic cells [7,98]. Suppression of *SH2B3* by CRISPR-Cas9 in primary human hematopoietic stem and progenitor cells enhanced the maturation and yield of in-vitro-derived RBCs [31]. This study suggests a new application of CRIS-Cas9 technology in improving cell and tissue production for regenerative medicine.

6. Summary and outlook

Over the last few years, the development of several gene editing technologies, ZFN, TALEN, and CRESPR-Cas9, has greatly promoted biology research and clinical trials for gene therapy. Among these three biotechnologies CRESPR-Cas9 is the latest player in the field but received great attention and applications. The main advantage of CRISPR-Cas9 is its simplicity, efficiency, and versatility for gene manipulations.

Targeting efficiency is one of the most important parameters for assessing a genome-editing tool especially the one used for gene therapy. Although a number of reports have confirmed that CRISPR-Cas9 has better targeting efficiency than ZFNs and TALENs, the efficiency still varies with cell types and research labs. For example, in zebrafish the targeting efficiency by CRISPR-Cas9 system has reached to 100% [46] and in one-cell mouse embryo 78% [108]. In contrast, in human CD4 + T cells the Cas9 system obtains efficiencies ranging from 5% to 20% [71,86] and in induced pluripotent stem cells only 2–5% [70]. Therefore, further improvement of the target efficiency and stability in hematopoietic cells is necessary. Moreover, it is unclear whether and how CRISPR-Cas9 is able to target *CCR5* or *CXCR4* effectively in all CD4 + T cells in vivo.

A number of papers described CRISPR/Cas9 off-target incidents but show somewhat conflicting results. Such incidents (mutations) usually occur at the sites where the nucleotide sequences are similar to the target sequences. If a foreign DNA, such as HIV DNA (reverse transcribed from the RNA), has very low homology with the host DNA, the off-target incidents may be not a big concern. Therefore, exhaustively screening the foreign DNA sequences and carefully designing gRNA can improve the off-target incidents. Recently, web-based tools have been developed to evaluate the possibility for off-target incidents and to help identification of CRISPR-Cas target sites [42].

In light of initial successful deletion of *CCR5* and *CXCR4* in T cells by CRISPR-Cas9 in HIV functional cure, it is necessary to continue this research on both specificity and efficiency. After all, these studies are still in the early stages of the development for the gene therapy. Based on current research, targeting *CCR5* and *CXCR4* in iPSCs derived from HIV patients and targeting the HIV genome are better strategies to cure HIV. However, eradicating all HIV, especially the latent reservoir is a big challenge. For hemoglobinopathies, i.e. β-thalassemia and SCD, the ultimate goal is to produce high quality iPSCs-derived from patients and their mutations have been efficiently corrected. These corrected iPSCs should be also able to differentiate into mature erythrocytes expressing high-level and normal HBB. Thus, a combination of genomic editing and stem cell transplantation would be the best strategy for basic research and clinical trials, which could replace traditional allogeneic stem cell transplantation. This strategy has several advantages: iPSCs can be obtained and prepared readily from patients and expanded as many times as required in vitro. Most importantly, it avoids possible graft rejection and risk to patients when these corrected iPSCs are returned to the patients. In order to increase targeting efficiency, careful design and select guide RNA is important. The safety issue of transplantation also needs to be studied carefully before corrected iPSCs are applied to patients. The initial success in targeting other genes in hematopoietic cells for identification of their functions projects broad prospects for applications of this technology for many other gene manipulations in hematopoietic systems including a possible cure of leukemia in the future.

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