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CRISPR/Cas9 system in hematopoietic stem cells: Basic research and clinical applications

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Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR) approach adapted from the prokaryotic adaptive immunity system against pathogen attack is so valuable and promising tool for treatment of human malignant and non-malignant hematological disease and disorders through gene editing in hematopoietic stem cells (HSCs). Moreover, the CRISPR/Cas9 approach is not only useful for therapeutic purposes; it is considerably preferred for the generation of in vitro and in vivo animal disease models. CRISPR/Cas9 approach has been developed for highly efficient on-target cleavage, and low off-target effect via delivery systems and manipulation of CRISPR components including single guide RNA (sgRNA) and Cas enzymes. In this review, we discussed the CRISPR/Cas9 system applications on hematopoietic stem cells in basic research and clinical area with basic research and clinical perspectives.

Keywords: hematopoietic stem cells, CRISPR/Cas9, gene editing, genome engineering, hematologic diseases, hematologic malignancies

1. Introduction

1.1. CRISPR System as Prokaryotic Adaptive Immunity System

The CRISPR system is found in prokaryotic organisms as a natural defense system against viral infection and exogenous plasmids. CRISPR system was discovered in Escherichia coli in 1987 (1) and it was found that this system cooperates with CRISPR-associated (Cas) proteins. We know that the prokaryotic organisms have a high ability for survival against to many difficult environmental conditions and viral attacks in spite of their simple cellular structure. This ability is associated with the management of genome homeostasis and protection from viruses using many defense systems (2). The CRISPR system, which is one of the defense systems, is mainly used against viruses, called as bacteriophages, in a prokaryotic cell. This system works in three stages including specific sequence recognition, targeting and degradation of exogenous foreign nucleic acid, respectively. CRISPR system is based on the recognition of the specific features of pathogens and thus, reminiscence of this pathogen features such as recognition of pathogen-derived structures by human immune memory B cells and rapidly reaction against to pathogens by adaptive immune system cells (3). The recognition occurs through the addition of the nucleic acids of viruses and exogenous plasmids into the CRISPR locus. This locus consists of short palindromic repeated sequences (25–35 bp). These sequences are separated by spacers (typically 30–40 bp each), also called as CRISPR array, and also the cluster of Cas genes (4). The spacer sequences of the CRISPR array that belong to different viruses provide targeting of the viral nucleic acids and destroy them when the viral attack is repeated. The insertion of the viral nucleic acid parts into the CRISPR array is also called as adaptation stage (2). In the adaptation stage, Cas protein complex first interacts with the target nucleic acid by recognition of the specific short (2–4 bp) sequence (known as protospacer-adjacent motif (PAM)) and then, double stranded breaks (DSBs) are generated in the target DNA. The ejected region of the target DNA is inserted into the CRISPR array (4). The adaptation stage is followed by the generation of a long precursor CRISPR RNA (pre-crRNA), also called as expression stage, through the transcription of the CRISPR array and expression of Cas genes. After the maturation of pre-crRNA by Cas proteins, the recognition of target viral nucleic acid results in the destruction of the target by crRNA and Cas endonucleases functions. Mature crRNA has a guide function to recognize and then cleave the foreign target nucleic acid which has similarity to the previously memorized sequences in CRISPR array (2, 3). Therefore, the crRNA is referred as guide RNA (gRNA) (4). This process is also called as RNA-mediated interference (2, 3).

Cas proteins encoded by Cas genes located in the CRISPR array are another essential components of CRISPR–Cas system. Cas proteins, which display effector role, are responsible for CRISPR/Cas system diversity. CRISPR/Cas systems are mainly grouped into two classes (Class 1 and Class 2) based on the structure of the effector complex and these

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classes consist of six major types (Type I-VI) based on the Cas protein variety and the CRISPR locus structure (5). The Class 1 consists of multi-protein effector complexes (Cascade, Cmr, Csm), and includes types I, III and IV. However, Class 2 contains effector proteins with a single subunit that promote effector complex functions and also includes types II, V, and VI (6). Type I in Class 1 is the most common system rather than other types. In Type I system, the targeting of DNA is promoted by Cascade and PAM-dependent manner and the target DNA is destroyed by using Cas3 protein. Besides, Type III system in Class 1 which is commonly found in archea, includes the multi-protein Csm or Cmr complexes. Thus, it promotes the recognition of foreign DNA or RNA regardless of the PAM sequence and the cleavage of targets by using Cas10 protein along with effector nucleases such as RNases Cmr4 and Csm3. The last system Type IV is rare system rather than the others (6, 7). In Class 2, Type II system is characterized by Cas9 endonuclease which is a multi-domain protein (7) and dual crRNA– transactivating crRNA (tracrRNA) guides which promote the guidance of the RuvC and HNH nickase domains to form the blunt-ended DNA DSBs in target DNA with 3′ PAM. Type V is rare system and includes Cpf1 (Cas12a) nuclease which promotes guidance of a single crRNA to RuvC-like endonuclease for generation of sticky-ends in target DNA with a 5′ PAM (6). The type VI CRISPR-Cas system, which encodes the HEPN domain (higher eukaryotes and prokaryotes nucleotide)-containing effector protein Cas13 (8), targets single-stranded RNA (ssRNA) with an RNase activity through the requirement for a protospacer flanking sequence (PFS) instead of the PAM sequence. Among Class 2, Types II and V are used for DNA editing, whereas type VI is used for RNA editing (9).

The diversity on CRISPR/Cas system leads to the diversity on its functions. CRISPR/Cas systems along with these properties have become so valuable for the development of new genome engineering tool. In particular, the CRISPR–Cas9 system among overall systems has become prominent with its simplicity (5) and has become the apple of the biotechnology.

1.2. CRISPR System as Genome Editing Tool

Genome editing has paved the way in the field of biotechnology by allowing for genetic manipulation. The development of genome editing tools provides the improvement of the treatment of monogenic diseases and disorders. Genome editing technologies are not only studied in animal and human cells but also, they are valuable tools for plant genome editing enabling the improvement of crop and nutritional value, resistance to crop disease and management of the biotic and abiotic stress in many crop species (10).

Until today, several genome editing tools have been developed. The overall tools are based on the sequence-specific programmable endonucleases including meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR-Cas (11). These engineered nucleases provide genome editing through DNA repair mechanisms including non-homologous end-joining (NHEJ) or homology-directed repair (HDR) following the generation of the DSBs at the specific DNA site (12). These engineered nucleases are divided into two classes depending on the strategy of target DNA recognition. Meganucleases, ZFNs and TALENs enable the target DNA recognition with protein-DNA interactions and thus, are included in one class. The second class consists of CRISPR/Cas which provides the targeting to specific DNA site by a short RNA guide molecule (13). Therefore, meganucleases, ZFNs and TALENs are referred to the protein-based platforms while CRISPR/Cas system is referred to the RNA-guided targeting (14).

Let's briefly mention other engineered nucleases before deeply getting into the CRISPR/Cas9 system: Meganucleases are restriction endonucleases and have large recognition sites (13). ZFNs and TALENs are chimeric endonucleases and have fused two domains including the DNA binding domain and the FokI nuclease domain (11). ZFNs have a DNA-binding domain consisting of a tandem array of Cys2His2 zinc fingers while TALENs have a DNA-binding domain derived from the proteins found in Xanthomonas plant bacteria (15). Among them, ZFNs are more expensive technology rather than the others and also, they are difficult to design. Besides, they are known to cause cytotoxicity. In contrast to ZFNs, TALENs are not cytotoxic but, they have low off-target activity (16).

Genome editing is accelerating with the development of CRISPR/Cas system modifying the mechanism of the prokaryotic adaptive immune system. CRISPR/Cas system in biotechnology uses type II and Cas9 displays a vital role as an effector protein. The RNA-guided CRISPR-Cas9 system is originally derived from Streptococcus pyogenes bacteria and it does not require target-specific protein interaction. Its specificity depends on the guidance of 20 nucleotide length guide RNA sequence and Cas9 effector protein. Guide RNA hybridizes with target DNA sequences and also, Cas9 protein is responsible for the recognition of 5′-NGG-3′ sequences known as PAM (16). Cas 9 protein has six different domains including HNH, RuvC, REC I, REC II, Bridge Helix, PAM interacting domains. The nuclease domains that are responsible for cleavage of target DNA are HNH and RuvC domains. Besides, the Bridge Helix domain is associated with the cleavage activity. The Rec I domain is essential for binding to guide RNA while the function of the REC II domain remains unclear. The PAM-interacting domain plays a crucial role in binding to target DNA through contributing to PAM specificity (17). The CRISPR/Cas9 system, like other tools, benefits from natural DNA repair mechanisms such as NHEJ and HDR. NHEJ repairs the DSBs through relegation of the two DSB ends without any requirement for template and also, is used for gene suppression through the formation of insertion or deletion (Indel) mutations. HDR requires a homologous template for repair and thus, it is used for replacement of a mutated gene

and recovery of gene (13). Besides, the adaptation (spacer acquisition), expression (crRNA biogenesis) and interference stages of the CRISPR system in a prokaryotic cell are adapted in the engineered technology with the specific design of single guide RNA (sgRNA). Moreover, trans-activating crRNA is discovered as an anti-sense RNA and a cofactor for Cas9 nuclease and, it also contributes to processing crRNAs (15). Briefly, CRISPR/Cas9 system initiates with the binding of gRNA to Cas9 nuclease and thus, Cas9 protein is activated with the conformational changes. Cas9 protein screens the DNA sequences to find the target DNA sequences that can match PAM specific sequence and then, gRNA binds to the target region. Finally, Cas9 nuclease domains form the cleavage on the target DNA (17).

The CRISPR-Cas9 system is more applicable than protein-based nucleases with many advantages. Cas9 protein is a stable component for CRISPR/Cas applications however; other nucleases are newly synthesized depending on the target. CRISPR-Cas9 is a cheaper technology than the others. Importantly, multiple gene editing can be performed with CRISPR/Cas9 system through the use of different sgRNA specifically designed for the different DNA sites (18).

CRISPR/Cas9 system is mainly used with three strategies including the plasmid-based CRISPR-Cas9 system, transfer of Cas9 mRNA and sgRNA and also, transfer of Cas9 protein and sgRNA to the target cell. Overall strategies have been developed to increase the CRISPR-Cas9 efficiency and thus, focus on different points and have different advantages and disadvantages. Plasmid-based CRISPR/Cas9 system is developed to prevent multiple transfections and thus, single plasmid encoding Cas9 nuclease and sgRNA, which is specific to the target DNA site, is used in this strategy. However, plasmid transfer processes result in different challenges. Another strategy involves the transfer of Cas9 mRNA and sgRNA into the target cell. However, low stability of transferred mRNA can cause the failure of gene editing and thus, it is a major problem for this strategy. The direct delivery of Cas9 protein and sgRNA into the target cell has many advantages, including high stability, showing faster function and low immune response (18).

The efficiency of CRISPR/Cas9 technology not only depends on Cas9 and sgRNA forms but, it also significantly depends on the delivery methods including viral and non-viral delivery systems. Each system has different advantages and disadvantages. In viral system, viral vectors are used for the delivery of CRISPR/Cas9 components. The utilized viral vectors are mainly divided into two groups such as integrating (retroviruses and lentiviruses) and non-integrating vectors (adeno-associated viruses (AAVs), adenoviruses, and herpes viruses) (18). Among them, AAVs and Lentiviruses are more useful and popular depending on their non-pathogenicity, low immunogenicity, and high infection efficiency (19). However, the integration of viral vector into the host cell genome is the main handicap for this delivery system. Therefore, non-viral delivery systems including the physical delivery systems, microinjection, electroporation, hydrodynamic delivery, lipid transfection, and utilization of gold nanoparticles become more preferable (18).

The utilization of CRISPR/Cas9 system in biotechnology is limited by off-target and on-target issues. It is expected that CRISPR/Cas9 system having sgRNA and PAM sequences matching to target DNA site only perform the genetic manipulations on the interested site. However, CRISPR/Cas9 system can recognize the undesired DNA regions and cleave these regions. This circumstance is called off-target effect of CRISPR/Cas9 system. Off-target effects cause genomic instability and toxicity, functional destruction in genes, epigenetic alterations, cell death, transformation and accordingly carcinogenesis (18). For reduction of the off-target and increase of the on-target efficiency, many various strategies based on the main components of the CRISPR/Cas9 system such as sgRNA and Cas9 have been developed. These strategies include truncation of the 3′ end of sgRNA and the addition of two guanine nucleotides to the 5′ end of the sgRNA, optimization of Cas9-sgRNA concentration, replacement of wild type Cas9 nuclease with D10 nickase, which is a mutant version of Cas9 allowing cleavage of only one strand (also known as paired nicking strategy), generation of fused catalytically dysfunctional Cas9 with FokI nuclease domain (fCas9) leading to enhanced DNA cleavage specificity and also, co-transfer of chemically modified sgRNAs with Cas9 mRNA or protein. Among them, modification of sgRNA and Cas9-sgRNA concentration strategies may reduce on-target specificity along with minimizing off-target effect and thus, it requires a balance (20). CRISPR/Cas9 applications are also limited by the induction of DNA damage toxicity because of the trigger of CRISPR-induced DSBs to apoptosis (21). To overcome this, different Cas9 variants such as catalytically inactive endonuclease dead Cas9 (dCas9), which perform gene editing without the generation of DSBs and Cas9 nickase, which induces single-strand breaks rather than DSBs, have been developed. These variants may prevent this limitation through the elimination of the risks of DSBs (22, 23). In addition to the DNA-damage toxicity, immunogenic toxicity caused by anti-Cas9 antibodies and the antibodies against the AAV vector used for the delivery of CRISPR components can be seen as a problem for CRISPR/Cas9 applications. For that, researchers have investigated the different Cas9 orthologs and AAV serotypes that may be safer for gene editing through prediction of the binding strength to major histocompatibility complex (MHC) class I and class II (21).

Studies are still being conducted to improve the efficiency of CRISPR/Cas9 and to avoid its limitations. The CRISPR/Cas system is a promising and valuable tool for therapeutically treatment of viral infection, cardiovascular, hematologic and eye diseases, muscular dystrophy, neurological and immunological disorders and also, cancer therapy. The

CRISPR/Cas system is not only used for gene therapy but also, it is important for the generation of transgenic cell lines and animal models through the mimicking of various modifications of DNA. Besides, the CRISPR/Cas9 system is preferred for the manipulation of plant genomes for crop improvement (17).

In this review, we will specifically discuss the applications of CRISPR/Cas system, which has a wide application area, in HSCs and thus, improvements in the treatment of malignant or non-malignant hematological diseases and disorders with research and clinical perspectives.

1.3. Basic Research on CRISPR System in Hematopoietic Stem Cells

In 2013, the CRISPR/Cas9-mediated genome editing was firstly applied in the mammalian cells (24, 25). This milestone has accelerated the research on the treatment of various diseases with gene therapy and the generation of in vitro and in vivo disease models. Especially, gene editing applications in HSCs are highly curative approach for many hematological diseases and disorders.

Hematopoietic stem cell transplantation (HSCT) is a widely used approach for the treatment of many hematological diseases and malignancies with the ability to reconstitute the hematopoietic system. HSCT is divided into two categories, such as autologous transplantation, in which the patient's healthy blood stem cells are used, and allogeneic transplantation, which uses the stem cells from foreign healthy donors. The utilization of allogeneic HSCT has many limitations, such as the lack of a human leukocyte antigen (HLA)-matched donor, the risk of progressing graft versus host disease (GVHD) and infection depending on immune suppression. As a result, autologous transplantation is a viable alternative to allogeneic HSCT and has demonstrated efficacy in clinical trials for hematological diseases and malignancies, metabolic storage diseases, and immunodeficiency disorders with the development of gene therapy. The autologous transplantation of genetically edited HSCs provides more safety and efficiency for various genetic and oncological diseases (26).

The monogenic β-hemoglobinopathy diseases caused by mutations in the β-globin (HBB) gene, commonly consist of sickle cell disease (SCD) and β-thalassemia and are conventionally treated by HSC transplantation (27). However, these hemoglobinopathies could be ameliorated by CRISPR/Cas9 system-mediated gene correction in patient-derived HSCs. Gene editing on β-hemoglobinopathies with CRISPR/Cas9 system is based on gene correction, HBB gene insertion, and the disruption of genes suppressing the fetal hemoglobin (HbF). Among them, gene correction and gene insertion are more difficult strategies because of the quiescent properties of HSCs that are not eligible for HDR that occurs in the G2 phase of the cell cycle. Therefore, an NHEJ-based strategy is more applicable (28). However, Dever et al. showed that CD34+ HSPCs obtained from mobilized peripheral blood (mPB) can be genetically modified at the HBB locus through the utilization of Cas9 ribonucleoproteins (RNPs) along with recombinant AAV6 donor delivery in spite of their resistance and the enrichment of the cells might enhance the editing efficiency if it is combined with HSC expansion technologies such as small molecule drug utilization (29). Another study also used the enrichment strategy of targeted-HSPCs (hematopoietic stem and progenitor cells) to minimize inefficient HSC targeting and optimized a protocol by using CRISPR/Cas9 and recombinant AAV6 homologous donor delivery for improvement of the gene therapy for blood and immune system diseases and disorders (30). Moreover, researchers described the new method based on electroporation of RNPs instead of using lentiviral transduction to edit the genomes of murine and human HSPCs using the CRISPR/Cas9 system and thus, showed that this method improves the CRISPR/Cas9 applications on hematopoiesis and hematologic diseases with no requirement for the stages of lentiviral transduction, minimizing the risks of lentiviral integration in unwanted regions and also, no requirement of a mouse strain expressing Cas9 (31). Many studies have focused on the disruption of genes suppressing HbF such as BCL11A, KLF1, and ZBTB7A genes for HbF reactivation (32). In relation to that, Lattanzi et al. tried to optimize a new efficient protocol for CRISPR/Cas9 mediated gene editing on β-globin locus in mobilized CD34+ HSPCs by using plasmid or lentiviral mediated and DNA-free delivery methods and demonstrated that the plasmid-mediated delivery method had high cell toxicity and also, lentiviral-mediated delivery had a high level of off-target cleavage. However, RNA or RNP delivery had low off-target effect and toxicity on primary HSPCs, thus it could be used for gene editing (33). Another study developed an optimized protocol based on the editing of the BCL11A enhancer gene which is required for HbF repression by using modified synthetic sgRNA, SpCas9 protein (34). Besides, Samuelson et al. also investigated the new platform including multiplex CRISPR/Cas9-mediated gene editing on the BCL11A enhancer and the HBG promoter, which are required for HbF repression, in human HSCs. They showed that targeting and dual-editing on these genes provided the generation of HbF but, resulted in chromosomal rearrangement and thus, reported that this protocol had some safety concern resulting from the generation of chromosomal translocations in clinical usage despite HbF reinduction (35). Yen et al. developed a device (called TRIAMF) which is based on a filter membrane and provides cell permeabilization for the delivery of RNPs to HSPCs as an alternative to electroporation system. They reported that the usage of TRIAMF provided the in vitro HbF induction and also, protected the normal multi-lineage and engraftment potential in NSG mice [Non-obese diabetic (NOD) severe combined immune deficient (SCID) gamma mice]. Besides, the data showing that the erythrocytes derived from the engrafted edited HSPCs provided the maintenance of high level HbF induction until 20 weeks as a result of TRIAMF device support that this low cost and non-electroporated device

is so promising for delivery of RNPs into HSPCs (36). Moreover, the information about that the precipitation of α globin induces the apoptosis and death of erythroid lineages indicates the importance of the balance between of α and β globin subunits in treatment of β-thalassemia caused by the abnormalities on hemoglobin synthesis which consists of 2 pairs of α and β globin subunits. The study trying to ameliorate the β-thalassemia with genetically editing of the α-globin locus in HSPCs was based on the two strategies including downregulation of α-globin gene HBA2 and also, upregulation of β-globin gene by using Cas9 nickase allowing precise gene editing without InDel mutations. They performed the CISPR/Cas9 mediated gene editing on healthy HSPCs and then, on HSPCs of the patients with β-thalassemia. They showed that the edited healthy HSPCs had the long-term repopulation and multipotency capacities, as well as that CRISPR/Cas9 mediated gene editing provided the correction of α/β globin imbalance in HSPC-derived erythroblasts. This study revealed the novel approach on CRISPR/Cas9 application in HSPCs for β-thalassemia treatment (37). In relation to the balance between α and β globin chains, Mettananda et al. also used an alternative strategy based on knockdown α-globin expression through CRISPR/Cas9-mediated mutation generation on MCS-R2 gene, which is an enhancer of α-globin in human CD34+ LT-HSCs and they provided the knockdown of α-globin in erythroid cells generated by edited HSCs without any distruption on erythroid differentiation or off-target activity (38). SCD from β-hemoglobinopathies is also being investigated for CRISPR/Cas9 mediated gene editing. The study about the correction of SCD mutation used the RNP/ single-stranded oligodeoxynucleotides (ssODN)-based CRISPR system and revealed the efficient HDR-mediated correction of the mutation in SCD HSPCs and also, enhanced production of γ-globin and fetal hemoglobin (39). In another study, it was shown that the usage of high-fidelity (HiFi) Cas9 variant with sgRNA and ssODN provided the correction of HBB gene in CD34+ HSPCs derived from SCD-patient and also, the edited cells differentiated into normal erythroid cells having a normal level of hemoglobin. Besides, these edited HSPCs had the ability to engraft and maintain post 16 weeks of transplantation (40). CRISPR-mediated gene editing is also a curative approach against immunodeficiencies, hematologic disorders and malignancies in addition to hemoglobinopathies. There are many researches regarding this.

Wiskott-Aldrich Syndrome (WAS) caused by mutations in the WAS gene, which is associated with cytoskeleton of hematopoietic cells, is an X-linked severe primary immunodeficiency. CRISPR/Cas9 or ZFN systems provides the gene addition to K-562 cancer cells with WAS donor template (41). Rai et al. also developed a CRISPR/Cas9 platform based on the knock-in WAS complementary DNA (cDNA) in patient-derived CD34+ HSPCs and thus, provided the amelioration of WAS expression by using gRNA targeting WAS 5′UTR and an AAV6 vector (42).

The X-linked chronic granulomatous disease (X-CGD) from immunodeficiency syndromes was also genetically corrected to repair a mutation in CYBB gene by CRISPR/Cas9 system with HDR mechanism in CD34+ HSPC (43). Sweeney et al. also focused on gene therapy for X-CGD treatment and presented a CRISPR/Cas9-mediated HDR repair approach based on the targeted insertion of CYBB cDNA in X-CGD patient-derived HSPCs in combination with inhibitor of 53BP1, which is responsible for choosing NHEJ over HDR (44).

Severe combined immunodeficiency (SCID-X1) from X-linked disorders is caused by mutations in the IL2RG gene located on the X chromosome and is a target for gene editing. Pavel-Dinu et al. described a modified CRISPR-Cas9-AAV6 approach based on the integration of cDNA which results in the gene correction >97% of IL2RG mutations on CD34+ HSPCs derived from SCID-X1 patient compared to healthy donor-, PB- and umbilical cord blood (UCB)-derived CD34+ HSPCs and also, provided the clinical advantages with safety (45).

In a study about the immunodeficiency focused on the treatment of X-linked agammaglobulinemia (XLA) caused by a mutation in the Bruton's tyrosine kinase (BTK) gene through the integration of BTK cDNA into the 5′ end of BTK locus by using the HR mediated-CRISPR-Cas9 approach in mPB CD34+ cells and they showed the safety and efficiency of this approach for XLA treatment (46).

Friedreich ataxia (FRDA) caused by genetic abnormalities in the FXN gene is an autosomal recessive disorder (47). Researchers who reported that the HSPC transplantation can be used for the treatment of FRDA carried out a study based on the gene correction on CD34+ cells from a patient's peripheral blood with FRDA by using CRISPR/Cas9 system. They corrected the FXN gene in FRDA patients' CD34+ cells through the removal of GAA expansion with high efficiency, no cytotoxic effect in vitro or in vivo while the transplanted cells had engraftment and clonogenicity abilities (48).

Gomez-Ospina et al. firstly showed the application of CRISPR/Cas9 mediated gene editing for the treatment of Mucopolysaccharidosis type I (MPSI), which is a common lysosomal storage disease (LSD) from genetic disorders caused by lysosomal protein deficiencies and also, caused by iduronidase (IDUA) deficiency. They overexpressed IDUA protein using the CCR5 locus to deliver RNP and AAV6-mediated templates into human CD34+ HSPCs, revealing an efficient platform that allowed the edited HSPCs to provide the lysosomal protein while maintaining long-term repopulation and multi-lineage differentiation potential in the MPSI mouse model (49).

From metabolic disorders for which hematopoietic stem cell transplantation is a treatment option, Pyruvate kinase

deficiency (PKD) is caused by mutations in the liver and erythroid pyruvate kinase gene (PKLR). Fañanas-Baquero et al. used an RNP delivery-based CRISPR/Cas9 approach through rAAV6 transduction in human UCB HSPCs and showed the correction phenotype in erythroid cells derived from edited-PKD-HSPCs (49).

The CRISPR/Cas9 system is also used for gene knock-in, which is required for supplementation of a protein (also called protein replacement therapy) that is deficient because of a genetic defect for the treatment of many diseases. Pavani et al. developed an ex vivo editing platform to integrate the therapeutic transgenes into the genome under the transcriptional control of the α-globin promoter, which is a suitable locus for transgene knock-in, for enhancement of their expressions on human HSPCs. After knock-in of therapeutic transgenes, they demonstrated that erythroblasts derived from targeted HSPCs secreted the therapeutic proteins with maintained multi-lineage differentiation and long-term repopulation potential and thus presented the safety of the novel CRISPR-Cas9-based HSPC platform (50).

Moreover, CIRSPR/Cas9 approach is more beneficial for the contribution to immunotherapy. The study about the generation of resistance to immunotherapy targeting CD33 in normal hematopoietic cells demonstrated the efficient CRISPR/Cas9-mediated deletion of CD33 exon 2, which is responsible for expression of the V-set domain recognized by therapeutics targeting CD33, but not full-length CD33 to reduce the potential adverse effects caused by in vitro and in vivo disruption of the entire CD33 locus in hematopoietic cells and in immunodeficient mice (51). Another group also investigated the potential of CRISPR/Cas9 approach based on the NHEJ-mediated disruption of the CD33 gene to generate the resistance to CD33 CAR T therapy in normal HSPCs for leukemia treatment. They generated the CD33 knockout mPB-HSPC and thus, revealed that CD33 knockout HSPCs provided immune reconstitution post acute myeloid leukemia (AML) targeting with CD33 CART therapy (52).

The applications of CRISPR/Cas9 system are so common for human immunodeficiency virus (HIV-1) treatment. The study about the generation of HIV-1 resistance cells revealed that CRISPR/Cas9 with two sgRNA guiding SaCas9 (Staphylococcus aureus Cas9) that is known with the effective gene editing ability and ease of delivery provided the distruption on chemokine receptor 5 (CCR5) gene by using lentiviral delivery method in primary CD4+ T cells and human CD34+ HSPCs and thus, present an alternative approach for HIV-1 treatment (53). In addition, many studies have been carried out for the generation of CRISPR/Cas9 mediated-CCR5 ablation human CD34+ HSPCs to obtain the HIV-1 resistance cells (54-56).

Gene editing tools are important for the treatment of the hereditary disease Fanconi anemia (FA) which is caused by different mutations in 22 FA genes. Autologous edited-HSC transplantation is mostly curative therapy (57). CRISPR/Cas9 approach has been mostly investigated in fibroblasts or induced-pluripotent stem cells derived from patients (58-63). Moreover, gene correction in HSCs is another strategy for FA treatment. Roman-Rodriquez et al. showed the correction of FA phenotype through the generation of compensatory mutations on the coding frame of FA proteins, which is required for the FA pathway, in FA patient-derived HSPC by using NHEJ-mediated repair in their preclinical study (64).

The CRISPR/Cas9 system is also used for the generation of disease models. Regarding this, Jeong et al. successfully generated a leukemia model by using the CRISPR/Cas9 system through induction of the chromosomal translocation between the MLL and AF9 genes in human UCB-derived CD34+ cells via the delivery of CRISPR/Cas9 as RNPs using electroporation (65). Another group also generated the murine leukemia model through the induction of reciprocal translocation between MLL and AF9 genes in both mouse cell line and primary isolated HSPCs by using CRISPR/Cas9 approach with a dual-single guide RNA (66). To generate a leukemia model characterized by translocation between MLL and ENL genes (t[11;19]/MLL-ENL), researchers generated chromosomal rearrangements on these gene locus by using the CRISPR/Cas9 system. This lentiviral-mediated CRISPR/Cas9 delivery system provided the generation of leukemia model through transformation of human UCB-derived CD34+ HSPCs (67). Schiroli et al. presented SCID-X1 mouse model with the efficacy and safety of hematopoietic reconstitution arising from edited HSPCs through CRISPR/Cas9-mediated gene correction on IL2RG in human HSPCs (68). Another gene editing study on HSPCs was carried out to generate the GATA1 transcription factor expressed HSPC model. They provided the expression of GATA1 isoforms including long and short isoforms in neonatal cord blood-derived long-term (LT-HSCs) and short-term HSCs (ST-HSCs), and myeloerythroid progenitors (MEPs). Therefore, down syndrome associated with AMKL was modeled with editing of GATA1 short isoform in CRISPR/Cas9 edited-HSPCs (69).

The CRISPR/Cas9 approach has been improved for more efficient editing without adverse effects including a low level of on-target cleavage and a high level of off-target activity. Researchers have optimized and developed many protocols for this aim. In this context, Hendel et al. used chemically modified sgRNAs, which is specific to IL2RG, HBB and CCR5, to induce gene editing efficiency and reduce off-target effect in cell lines and primary isolated human T and CD34+ HSPCs. They revealed that chemically modified sgRNA enhanced genome editing efficiencies rather than unmodified sgRNA through co-delivery of chemically modified sgRNAs with Cas9 mRNA or protein (70). Mandal et al. investigated the on-target efficiency and off-target cleavage risks depending on the utilization of sgRNA and dual gRNA in the CRISPR/Cas9 system in primary isolated human CD4+ T and CD34+ HSPCs through generation of beta-2 microglobulin

(B2M) and CCR5 gene ablations. B2M, which is a component of MHC class I, ablation could be useful for generation of hypoimmunogenic cells for immunotherapy molecules and also, CCR5 ablation is important for the protection from HIV infection. They showed that the use of CRISPR/Cas9 with sgRNAs provided the efficient CCR5 ablation in CD34+ HSPCs, but not for B2M ablation in CD4+ T cells. However, the use of CRISPR/Cas9 with a dual gRNA enhanced gene deletion efficiency in CD4+ T and CD34+ HSPCs for B2M gene and also, for CCR5 gene in CD34+ HSPCs. This approach is promising for efficient on-target and low off-target mutagenesis (71). In this regard, a study on CCR5 ablation using CRISPR/Cas9 editing revealed that CCR5 was edited by a non-viral CRISPR/Cas9 system with minimal off-target effect, and thus CCR5 ablated LT-HSCs provided long-term reconstitution and improved resistance to HIV-1 infection in transplanted immunodeficient mice. These findings revealed an alternative strategy for gene therapy on HSCs (54).

1.4. Clinical Applications of CRISPR System for Hematopoietic Stem Cells

Hematopoietic stem cells are generally used for curative therapy as allogeneic or autologous HSCT for hematological malignant /non-malignant and monogenic diseases. As explained before, allogeneic HSCT has many limitations including lack of HLA-matched donor, risk of GVHD and infection arising from immune suppression occurring for transplantation. Herein, autologous HSCT presents an alternative approach for eliminating these adverse effects of allogeneic HSCT. Especially, autologous transplantation of CRISPR/Cas9 mediated-edited HSCs is so promising for many hematological and monogenic diseases and disorders. Gene therapy approach with viral vectors and gene editing tools such as ZFNs, TALENs and Meganucleases have recently been mostly preferable for treatment of many diseases. Especially, there are several ongoing and completed clinical trials about gene editing in HSCs (http://clinicaltrials.gov/) (summarized in Table 1). Among these, ZFNs-based gene editing in HSCs has been extensively clinically tested for inducing resistance to HIV infection via CCR5 disruption (NCT02500849) and reactivation of HbF for hemoglobinopathy treatment via BCL11A enhancer blocking (NCT03432364). The advantages and efficiency of CRISPR/Cas9 system have become a promising approach for gene correction in HSCs (72). Among them, CTX001, which is autologous CD34+ hHSPCs genetically edited by CRISPR-Cas9 system on BCL11A gene to produce HbF in HSCs, is particularly tested in phase I/II clinical trials in pediatric and general subject with β-hemoglobinopathies (β-thalassemia (NCT03655678; NCT05356195) and SCD (NCT03745287; NCT05329649). Another clinical trial (NCT04925206) has also been carried out for β-thalassemia treatment through targeting of BCL11A repressor gene. In addition to reactivation of HbF, the replacement of mutated β-globin through gene correction is another approach for treatment of β-hemoglobinopathies (β-thalassemia (NCT03728322; NCT05444894) and SCD (NCT04774536; NCT04819841). Other clinical trial has been carried out to investigate resistance to CD33-targeted-immunotherapy through the CD33 ablation with CRISPR system in HSCs (NCT04849910). Moreover, a clinical study (NCT03164135) has been performed for the evaluation of the safety of CRISPR-mediated CCR5 modified CD34+ cell transplantation for HIV-1 treatment.

Table 1. Clinical trials associated with CRISPR/Cas9-mediated gene editing in HSCs

NCT number	Phase	Disease	Target	Responsible party	Status
NCT03655678	II/III	β-Thalassemia (Pediatric Participants)	BCL11A	Vertex Pharmaceuticals Incorporated	Active
NCT04925206	I	β-Thalassemia	BCL11A	EdiGene (GuangZhou) Inc.	Active
NCT05356195	III	β-Thalassemia	BCL11A	Vertex Pharmaceuticals Incorporated	Recruiting
NCT04208529	LT-Follow-up Study	β-Thalassemia	BCL11A	Vertex Pharmaceuticals Incorporated	Enrolling by invitation
NCT03728322	Early Phase 1	β-Thalassemia	β-globin	Allife Medical Science and Technology Co., Ltd.	Unknown
NCT05444894	I/II	β-Thalassemia	β-globin	Editas Medicine, Inc.	Recruiting
NCT04774536	I/II	SCD	β-globin	Mark Walters, MD, Professor in Residence, University of California, San Francisco	Not yet recruiting
NCT04819841	I/II	SCD	β-globin	Graphite Bio, Inc.	Recruiting
NCT03745287	II/III	SCD	BCL11A	Vertex Pharmaceuticals Incorporated	Active
NCT05329649	III	SCD (Pediatric Participants)	BCL11A	Vertex Pharmaceuticals Incorporated	Recruiting
NCT04849910	I/II	AML	CD33	Vor Biopharma	Recruiting
NCT05309733	LT- Follow-up Study	AML	CD33	Vor Biopharma	Recruiting
NCT03164135	-	HIV-1	CCR5	Affiliated Hospital to Academy of Military Medical Sciences	Unknown

Note: Information from clinicaltrials.gov.tr; LT: Long-term, SCD: Sickle cell disease; AML: Acute myeloid leukemia, HIV-1: human immunodeficiency virus

In the light of all preclinical and clinical studies, it can be highlighted the power of CRISPR/Cas9 approach in therapeutic area and the importance of the improvement of new platforms to enhance the gene editing efficiency and clinically feasibility.

2. Conclusion

HSCs which are valuable for the treatment of malignant and non-malignant hematological disease and disorders via transplantation are offered as a target for gene editing. Therefore, the CRISPR/Cas9 system of genome editing tools has been developed as a curative approach for hematological malignancies and diseases. In spite of the many advantages of the CIRSPR/Cas9 system including its fast and cost-effective, this technology has several technical limitations. Many studies have developed new platforms and protocols to overcome these limitations. Until today, many preclinical and clinical studies have shown the potential of CRISPR/Cas9 approach in correction of genetic abnormalities in HSCs and the curative action of the CRISPR/Cas9-mediated edited HSCs. Along with all these properties, CRISPR/Cas9 gene editing tool is considered a valuable and promising therapeutic method for future gene therapy.

Conflict of interest

None to declare.

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Authors' contributions

Concept: E.A., Design: E.A., Data Collection or Processing: E.A., Analysis or Interpretation: E.A. Literature Search: E.A., Writing: E.A.

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