Advancement Of Gene Therapy in Blood Diseases Treatment Based On CRISPR/Cas System

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Abstract. Blood-related diseases are characterized as physiological disorders that affect the blood's functions, affecting tens of millions of people's lives every year. Serious blood disorders can be life threatening and bring patients both great physical and mental pain. Gene therapy has the potential to significantly intervene in disease from the standpoint of genetic material, resulting in a more beneficial therapeutic outcome. CRISPR/Cas system, as the third gene-editing technology, has become a powerful tool for gene editing. This review provided a brief summary of the current advancements, together with advantages, challenges of CRISPR/Cas system, and discussed future prospects of utilizing CRISPR/Cas system for genetic therapy in blood diseases.

Keywords: Blood Diseases; CRISPR/Cas system; gene therapy.

1. Introduction

Blood Diseases are physiological disorders that affect the blood's functions, including red cell disorders (e.g., sickle cell disease), white blood cell disorders (e.g., leukemia), etc. Hematopoietic stem cell transplantation (HSCT), as a classic treatment, facing the problem - the difficulty of matching a donor and requirement of immunosuppression. Immunotherapy mostly entails altering T-cells, B-cells, and other immune cells in order to better employ one's own immune system to combat disease. But general adoption of immunotherapy is still challenging.

CRISPR/Cas is the third-generation gene-editing technique after ZFN and TALEN, featuring high efficiency and precision, and its development started in 2012. The creators of the CRISPR gene editing system, Emmanuelle Charpentier and Jennifer A. Doudna, received the 2020 Nobel Prize in Chemistry. Utilizing CRISPR/Cas to perform gene therapy is an emerging medical technology. Gene therapy can be achieved through different methods, including inserting normal gene, or repairing or removing defective genes. In most cases stem cells are taken out and manipulated, because stem cells can be differentiated into target cells. Then the edited cells are transplanted back to the patient and have potential to cure the disease.

In recent years, CRISPR/Cas technology has been employed extensively in gene therapy for blood diseases. Herein, gene therapies using CRISPR/Cas of sickle cell disease and leukemia will be discussed, together with a few other diseases, followed by their prospect in curing.

2. CRISPR/Cas system

2.1. The principles of CRISPR/Cas-mediated genome editing

CRISPR/Cas9 system is the most commonly used system, which was first discovered in bacteria and archaea, mainly functioned as a way to combat infection of phage. CRISPR is an array of repeats and spacers that can be transcribed to form CRISPR RNA (crRNA). The repeats are identical palindromic sequences, which allows the crRNA to form a hairpin-like structure in the corresponding regions. The bacteria or archaea will record the DNA fragments of the invading phage by inserting it into the spacer sequence. When the phage invades again, crRNA are transcribed, with the "spacer" matching the DNA sequence of the invading phage.Then the crRNA binds with Cas protein and guides it to cut the DNA double strand of phage, thus defending against its invasion. There is also a trans-activating crRNA (tracrRNA) to fix the crRNA in the correct binding position with Cas9.

Moreover, crRNA and tracrRNA are artificially modified and merged into one to form single guide RNA (sgRNA), which is used to target specific DNA sequence in gene manipulations (Figure 1).

The function of Cas proteins is to induce double strand break (DSB). Cas9 protein contains two domains to cut the DNA strand - HNH and RuvC. The guide RNA-bound target DNA strand is cut by the HNH nuclease domain, whereas the complementary DNA strand is cut between 3 and 4 bases upstream of PAM by the RuvC nuclease domain. After the DSB happens, the DNA can be repaired by methods such as homology-directed repair (HDR) or nonhomologous end joining (NHEJ). For the most cases, after NHEJ the original gene sequence is altered and damaged, caused by base pair insertions and deletions (indels), resulting in gene knockout (KO). By using the mechanism of HDR, we can knock in (KI) specific genes, too.

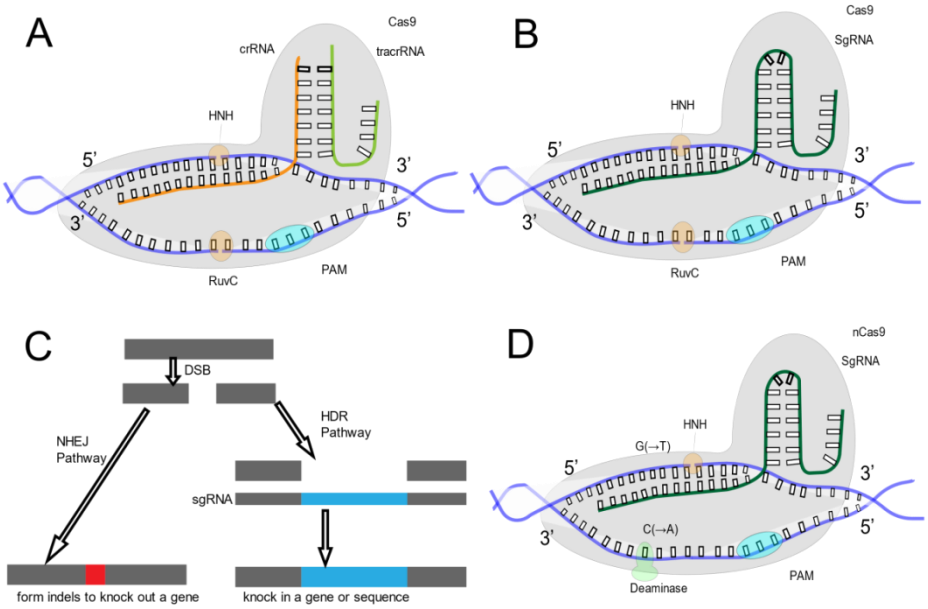


Fig. 1 The process of CRISPR/Cas9 system.

The wild process of CRISPR/Cas9 in a bacteria self-protection. B) the process of CRISPR/Cas9 in gene-manipulation. C) NHEJ and HDR pathways for gene repairing. D) The process of cytosine base editor.

2.2. Types of CRISPR/Cas gene editing tools

By modifying the structural domain of Cas9, it is possible to obtain Cas9 nickase (nCas9), which can only cut one of DNA strands, and dead Cas9 (dCas9), which has no cutting function. A number of single-base substitution tools, including cytosine base editor (CBE), adenine base editor (ABE), and prime editor (PE), have been developed. They can combine with dCas9 or nCas9 to edit specific base pairs in target site within the DNA. For example, CBE is a combination of cytidine deaminases with the nCas9, and enables targeted conversions from C to T, while ABE catalyzes specific A-to-G conversions. Prime editing was achieved by using nCas9 and doing reverse transcription, with the help of pegRNA as the template and regulatory sequence for editing [1].

3. Therapy based on CRISPR/Cas system

3.1. CRISPR used in sickle cell disease therapy

Sickle cell disease (SCD) is a widely known monogenic inherited disease caused by an abnormal structure of hemoglobin, leading to reduced ability to transport oxygen. SCD patients show symptoms like anemia, red cells lackage and severe fatigue. Among the diseases which CRISPR gene therapy

is currently developed for, results of SCD are relatively favorable. There are two main categories - by taking out and editing target cells ex vivo before returning them to the patient, or by directly delivering CRISPR-Cas editing tools to the target tissue, in vivo (Figure 2).

Fetal hemoglobin (HbF), also having the ability of transporting oxygen like β hemoglobin, is expressed just in infancy and later suppressed from expression. The inhibition of HbF expression can be removed by editing and disabling the binding site of the gene-expression repressor. Martyn et al. found that BCL11A and ZBTB7A, two of the main fetal globin gene repressors, directly bind to certain upstream sites of the HbF gene [2]. Humbert et al. used CRISPR-Cas9 to edit long-lived stem cells to knock out BCL11A binding site. In this way, normal ability of oxygen transport can be restored and compensate for the abnormal function of β-hemoglobin. They also did experiment on rhesus macaques, a nonhuman primate, and observed ideal results after the transplantation of edited cells [3].

Newby et al. performed mice experiment. In haematopoietic stem and progenitor cells (HSPCs) from SCD patients, about 80% of SCD β-hemoglobin genes were effectively converted to benign β-hemoglobin genes by ex vivo administration of mRNA encoding the base editor together with a targeting guide RNA. The proportion of benign β-hemoglobin genes was 68% and the hypoxia-induced sickling of bone marrow reticulocytes decreased fivefold, 16 weeks after the transplanting of altered human HSPCs into immune-deficient mice, which demonstrates lasting effect of gene editing. Normal β-like globins (βG) appears and constitutes 76% after mRNA editing, and proportion of sickled cells decreased significantly after editing [4].

Frangoul et al. performed clinical trial. The patient with SCD received the CTX001 treatment which means that using CRISPR-Cas9 to edit CD34+ cells (stem cells) to enhance the expression of BCL11A, thus increase the amount of fetal hemoglobin (HbF). Five months after transfusion of CTX001, the proportion of HbF increased from 9.1% to 48.6%, and could remain stable for more than 10 months after that [5].

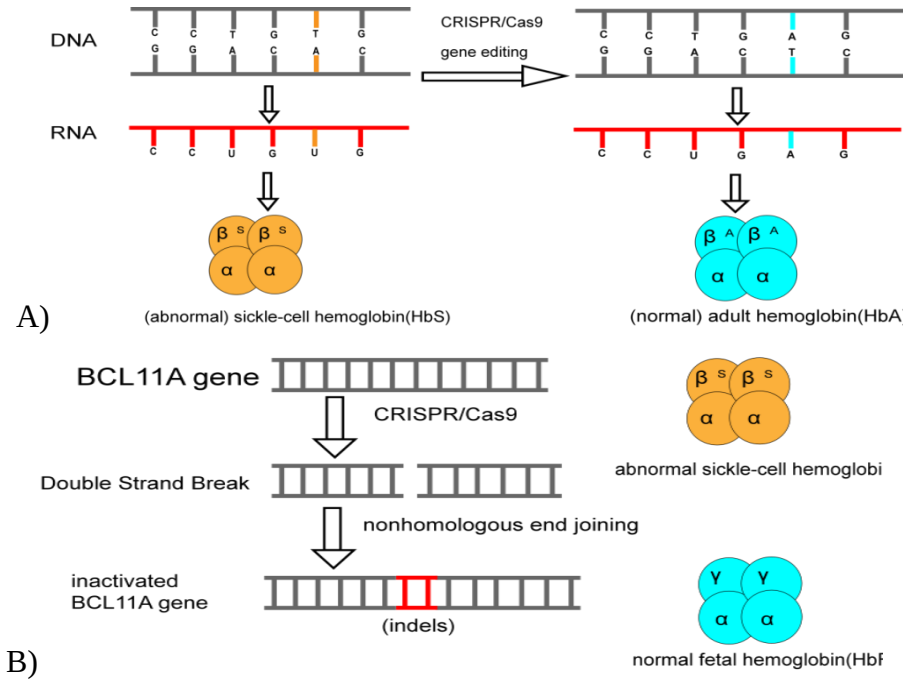


Fig. 2 Two methods to treat SCD.

A) knock out BCL11A to express HbF and B) directly modify the Hb sequence.

All in all, CRISPR technology has been explored as an effective approach to correct the genetic mutation responsible for abnormal hemoglobin. Nevertheless, long-term safety and efficiency studies are needed when doing further clinical trials and preparing for wider applications.

3.2. CRISPR used in leukemia therapy

Leukemia, a widely known disease, refers to cancer of the blood-forming tissues - bone marrow and lymph. It can be classified into acute and chronic leukemia according to its progression speed or be classified into lymphocytic and myelogenous leukemia according to type of affected white blood cells. Chemotherapy is a common treatment for leukemia but can harm normal blood cells. Bone marrow transplantation is also commonly used as a treatment, but it also creates a series of subsequent problems, such as the need for immunosuppression.

Table 1 Types of leukemia.

Sort of leukemia	onset age	new patients US (2023)	new deaths US (2023)	conventional therapy	CRISPR usage	Ref.
ALL (acute lymphocytic leukemia)	mainly < 5, or > 50	6540	1390	chemotherapy,stem cell transplants, targeted drugs	assist CAR-T therapy, etc.	[6]
AML (acute myeloid leukemia)	>45, on average 68	20380	11310	chemotherapy,stem cell transplants, targeted drugs	assist CAR-T therapy, CRISPR screens, etc.	[7]
CLL (chronic lymphocytic leukemia)	on average ~70	18740	4490	targeted drugs	target and repair BCR-ABL junction, etc.	[8]
CML (chronic myeloid leukemia)	on average ~64	8860	1220	targeted drugs	target and repair BCR-ABL junction, etc.	[9]

CRISPR screen can identify promising therapeutic targets, which effectively promote new drug development. Bajaj et al. utilized CRISPR screen technology to search for essential regulators associated with chronic myeloid leukemia (CML). After blocking thousands of genes, they found that Staufen2 (Stau2), one of RNA-binding proteins (RBPs), is a key regulator of CML. Additionally, they discovered that in mice models, removing Stau2 significantly reduced the development of leukemia cells., proving its importance [10].

Quijada-Álamo et al. used CRISPR/Cas9 system for making in vitro chronic lymphocytic leukemia (CLL) models immitating typical CLL genomic alterations, including deletion of chromosome 11q22.3. By using these models, they probed the effects of these genomic alterations on the response of DNA damage which signals CLL [11].

Improving CAR-T therapy with CRISPR is a promising therapeutic idea in recent years. Chimeric antigen receptor (CAR)-T cells can kill cancerous cells efficiently. Kim et al. developed a method to modify hematopoietic stem and progenitor cells (HSPC) with CRISPR. They used CRISPR/Cas9 to knock out the CD33 gene in HSPC, whose resulting blood cells no longer express CD33 protein on their surface. But leukemia cells still express CD33, and thus are killed by CAR-T cells, with normal cells unaffected. Animal experiments showed that the modified HSPCs could survive stably and constantly, and their physiological functions were not affected after differentiation [7]. The clinical trial named “VBP101” also used the method of knocking out the CD33 gene in HSPC.

One of the most prevalent types of adult leukemia is (CML), and the well-known medicine Gleevec is often used for its treatment. The pathogenesis of CML is a chromosomal translocation that produces a BCR-ABL fusion gene, leading to changes in kinase activity. Chen et al. used CRISPR/Cas9 to gene edit human CML leukemia cell K562 to knock out its ABL gene. In the in vitro experiment,

they found that ABL loss curbed the growth of K562 cell. In the in vivo experiment, they injected K562 cells into the mice and injected CRISPR gene editing tools in some of them. The results showed that K562 cell development was noticeably slowed down in the mice after gene editing, measured by fluorescent protein imaging. Edited K562 cells showed higher rate of apoptosis compared to unedited cells, due to lack of normal ABL proteins [8].

All in all, it can be seen that CRISPR technology has important applications in both mechanistic studies and treatment of acute or chronic leukemia. In the future, it will also be more combined with immunotherapy as a potential means of leukemia treatment.

Some clinical trials of CRISPR used in SCD and leukemia treatment.

Table 2 clinical trials of CRISPR used in SCD and leukemia treatment.

Publish Year	time period	age and gender	illness	Target sequence	Method	Result	Ref.
2021	15 months	33, female	SCD	BCL11A	CTX001	HbF grew to >91% of total Hbs, no vaso-occlusive and do not need transfusion	[5]
2023	18 months	22, male	SCD	HBG1 and HBG2	OTQ923	more HbF and relevant cells, alleviated symptoms	[12]
2023	12 months	21, male	SCD	HBG1 and HBG2	OTQ923	more HbF and relevant cells, alleviated symptoms	[12]
2023	6 months	24, female	SCD	HBG1 and HBG2	OTQ923	more HbF and relevant cells, alleviated symptoms	[12]
2022	5 months	25, male	SCD	HBG1 and HBG2	AsCas12a	more HbF and relevant cells, no vaso-occlusive events	*
2022	1.5 months	31, female	SCD	HBG1 and HBG2	AsCas12a	no vaso-occlusive events	*
2019	~19 months	27, male	ALL	CCR5 gene	-	complete remission after transplantation	[13]
2023	~4 months	(8 patients)	AML	CD33 gene	-	maintained neutrophil and platelet counts, low side effects	**

*: some of examples from clinical trial named “EDIT-301”, a big programme which is targeted to cure SCD or TDT using Cas12a gene therapy. Data are from the internet.

**first successful trial from clinical trial named “VBP101”, which is conducted by Vor Bio. Data are from the internet.

3.3. CRISPR used in other blood diseases

3.3.1 β-thalassemia

β-thalassemia (TDT) is a monogenic hemoglobin (Hb) disorder. It is brought on by mutations that might result in decreased or lost synthesis of the β-chains of hemoglobin. This uneven ratio of α-chain to β-chain globin causes several disorders like microcytic anemia and disruptions in the generation of red blood cells (RBCs). The conventional therapy of it mainly includes frequent blood transfusions, stem cell transplantation and removing excess iron from the blood. One of its effective gene therapy strategies is also to reactivate fetal hemoglobin (HbF) expression.

The clinical research into two juvenile patients performed by Fu et al. used CRISPR/Cas9 to edit the enhancer of BCL11A into the stem cells. As a result, their Hb increased from 8.2 and 10.8 g/dl to 15.0 and 14.0 g/dl, together with editing persistence of 85.46% and 89.48%, respectively. Following treatment, neither patient required a transfusion for longer than 18 months, and the treatment had no significant side effects proved by exploratory analysis [14].

3.3.2 Atherosclerosis

Atherosclerosis is a common disease occurring when the arteries become narrowed and hardened as a result of plaque (fats) accumulated in the artery wall. It is always the consequence of high levels of low-density lipoprotein (LDL) cholesterol, marked by the existence of fibro-fatty lesions in artery. CRISPR/Cas9 can be utilized to generate atherosclerosis disease models, both in vivo and in vitro. To treat the disease, gene targets like PCSK9, APOC3, and ANGPTL3 can be inactivated [15]. Musunuru et al. delivered CRISPR base editor encapsulated in lipid nanoparticles which are then sent to the cynomolgus monkeys. It successfully modified disease-related genes, and PCSK9 was nearly completely knocked down. The levels of PCSK9 and LDL decreased concurrently by 90% and 60% respectively, better than drugs such as statins. After a single dose of medication, each change stayed constant for more than eight months [16].

3.3.3 Hemophilia

Hemophilia A and B are X chromosome-linked inherited disorder, meaning that the blood is unable to clot after injury, resulting from lack or abnormal function of either blood coagulation factor VIII (type A) or factor IX (type B). Transfusion of clotting factor or healthy plasma is a way to stop patients from bleeding, but the HA patients require repetitive FVIII injections. In one kind of hemophilia A, there is a reversal of the promoter and exon 1 included in coagulation factor VIII gene, resulting in its inability to be expressed properly. Hu et al. used CRISPR/Cas9 to knock in the promoter and exon 1 fragments of coagulation factor VIII. They succeeded in gene editing of stem cells, and the differentiated cells could express coagulation factor VIII normally [17]. There are several clinical trials of therapy based on CRISPR/Cas9 technology ongoing and show positive results. After gene therapy, the patient can get a certain level of coagulation factors, which help them to coagulate blood normally.

4. Advantages and Challenges of therapy based on CRISPR/Cas system

4.1. Advantages

In comparison to other gene-editing techniques, zinc-finger nucleases (ZFN) and transcription activator like effector nucleases (TALEN), the CRISPR/Cas9 system offers a number of benefits. For example, ZFN and TALEN can only modify a few DNA sites, but CRISPR can be used at more available sites and nearly every gene. In about every 8 bases, there is a site that can be edited with CRISPR/Cas9. Additionally, nCas9 and dCas9 can significantly expands its applications. ZFN and TALEN technologies need specific DNA-binding proteins that are carefully engineered for each DNA target location. By contrast, CRISPR/Cas system relies on the guide RNA to target the DNA site. ZFN has cell toxicity, but CRISPR/Cas system is relatively safe for in vivo and in vitro therapy.

4.2. Challenges

The use of CRISPR in treating blood diseases holds potential but faces various challenges. Off-target effects, delivery methods, and effectiveness remain obstacles to overcome. Additionally, the ethical implications of gene editing require careful consideration.It is crucial to pick the ideal delivery method for introducing the CRISPR system into cells and to target on the correct nucleus sequences, especially in vivo.

4.2.1 Off-target effects

Off-target effect is considered the main concern or CRISPR gene editing. The off-target effect means that the gene editing tool edits DNA sequences that is not expected to be edited. The sgRNA may bind partly with off-target sites of DNA with non-canonical base-pairing interactions [18]. Off-target effects can make abnormal functions of other genes. There are several methods to detect possible off-target effects, including methods used in silico and in vitro. For the purpose of minimizing off-target effects and genomic rearrangements, several changes have been created. This includes sgRNA modifications (e.g. 2'-O-methyl-3'-phosphonoacetate), Cas9 protein optimizations (e.g. designing artificial Cas9 types such as eSpCas9, FeCas9), etc. However, some of these methods decrease on-target activity as well.

4.2.2 Delivery methods

Another challenge is figuring out how to get the gene-editing tools into the cell. Cas9 and sgRNA can be delivered into the targeted cell primarily in three ways: by viral vectors, nanoparticles, and electroporation of protein-RNA complexes. The most often utilized viral vectors are Adeno-associated virus (AAV), which has been used in curing a series of diseases like spinal muscular atrophy and congenital blindness. AAVs use their genome to carry the sequence of Cas9 and sgRNA. However, the limited length of genes that AAV can carry (4.7kb) makes it difficult to introduce the Cas9 coding sequence (4.2kb), sgRNA, and other regulatory sequences into the target cell simultaneously. The use of smaller gene-edited proteins can alleviate such problems to some extent. Notwithstanding, unexpected immune reactions may happen if genome-editing molecules remain expressing for too long time. The delivery via nanoparticles is employed in a variety of therapeutics for disorders such as inherited blindness and can reduce some of safety problems brought by viral vectors [19].

4.2.3 Effectiveness

The effectiveness of gene-editing is also a problem. For example, HDR after CRISPR/Cas9-caused DSB has been employed in targeted knocking in DNA sequence. But other repairing ways such as NHEJ can happen before HDR, thereby declining the effectiveness. Increasing the efficacy of HDR may be accomplished in a number of ways, including by blocking the NHEJ pathway, controlling HDR-related factors, and choosing the right donor DNA template. For example, Arai et al. used new manipulation methods to increase the effectiveness of a plasmid donor's knock-in using HDR and suppress other repairing ways strongly [20].

5. New developments

5.1. Utilizing Machine Learning Techniques

Machine learning and artificial intelligence are gradually emerging in CRISPR gene-editing with many new applications. For example, high throughput screening can be used to decide the potential editing point within the gene sequence. Machine learning can be used to devise sgRNA and evaluate its combining effect with genome, and decrease the possibility of off-target, by using former studies as datasets. Kim et al. developed a model named DeepSpCas9, which can be used to predict the activity of SpCas9 with high throughput [21]. Cheng et al. designed a deep learning model DeepCas13, to predict the on-target activity of CRISPR-Cas13d from guide sequences and secondary structures [22].

5.2. New kinds of CRISPR-like systems

The CRISPR system originates from prokaryotes such as bacteria. Many different CRISPR proteins have been discovered and applied to fulfill different functions. However, scientists are always looking for new types of CRISPR proteins to adapt to different contexts. For example, recently, one of CRISPR founders Feng Zhang's team discovered Fanzor, the first RNA-guided DNA-cutting

enzyme in eukaryotes. This new CRISPR-like system enables editing of genome after reprogramming. The team also analyzed the molecular structure of the Fanzor-ωRNA complex and resolved how it attaches to and cleaves DNA [23].

6. Conclusions

Progress in CRISPR/Cas systems happened in the past 10 years, opening the door for developments in fundamental study and medical applications. Nevertheless, further research and development are essential to address the challenges and ensure its safety and efficacy in clinical applications.

In this review, recent research advances related to CRISPR technology in the treatment of blood diseases were discussed, especially for sickle cell disease and leukemia. In the future study, there will be more robust and reliable new methods in CRISPR technology. It will hold more potential to impact the treatment landscape for treating diseases that are otherwise hard to cure, offering hope for patients worldwide. Scientists, doctors, bioethicists, economics, and regulators need to collaborate in order to achieve the potential of safe, efficient, and cheap results of gene therapy.

References

[1] Li, T., Yang, Y., Qi, H., Cui, W., Zhang, L., Fu, X., ... & Yu, T. (2023). CRISPR/Cas9 therapeutics: progress and prospects. Signal Transduction and Targeted Therapy, 8(1), 36.

[2] Martyn, G. E., Wienert, B., Yang, L., Shah, M., Norton, L. J., Burdach, J., ... & Crossley, M. (2018). Natural regulatory mutations elevate the fetal globin gene via disruption of BCL11A or ZBTB7A binding. Nature genetics, 50(4), 498-503.

[3] Humbert, O., Radtke, S., Samuelson, C., Carrillo, R. R., Perez, A. M., Reddy, S. S., ... & Kiem, H. P. (2019). Therapeutically relevant engraftment of a CRISPR-Cas9–edited HSC-enriched population with HbF reactivation in nonhuman primates. Science translational medicine, 11(503), eaaw3768.

[4] Newby, G. A., Yen, J. S., Woodard, K. J., Mayuranathan, T., Lazzarotto, C. R., Li, Y., ... & Liu, D. R. (2021). Base editing of haematopoietic stem cells rescues sickle cell disease in mice. Nature, 595(7866), 295-302.

[5] Frangoul, H., Altshuler, D., Cappellini, M. D., Chen, Y. S., Domm, J., Eustace, B. K., ... & Corbacioglu, S. (2021). CRISPR-Cas9 gene editing for sickle cell disease and β-thalassemia. New England Journal of Medicine, 384(3), 252-260.

[6] Hu, Y., Zhou, Y., Zhang, M., Ge, W., Li, Y., Yang, L., ... & Huang, H. (2021). CRISPR/Cas9-engineered universal CD19/CD22 dual-targeted CAR-T cell therapy for relapsed/refractory B-cell acute lymphoblastic leukemia. Clinical Cancer Research, 27(10), 2764-2772.

[7] Kim, M. Y., Yu, K. R., Kenderian, S. S., Ruella, M., Chen, S., Shin, T. H., ... & Gill, S. (2018). Genetic inactivation of CD33 in hematopoietic stem cells to enable CAR T cell immunotherapy for acute myeloid leukemia. Cell, 173(6), 1439-1453.

[8] Chen, S. H., Hsieh, Y. Y., Tzeng, H. E., Lin, C. Y., Hsu, K. W., Chiang, Y. S., ... & Lee, C. H. (2020). ABL genomic editing sufficiently abolishes oncogenesis of human chronic myeloid leukemia cells in vitro and in vivo. Cancers, 12(6), 1399.

[9] Vuelta, E., García-Tuñón, I., Hernández-Carabias, P., Méndez, L., & Sánchez-Martín, M. (2021). Future approaches for treating chronic myeloid leukemia: CRISPR therapy. Biology, 10(2), 118.

[10] Bajaj, J., Hamilton, M., Shima, Y., Chambers, K., Spinler, K., Van Nostrand, E. L., ... & Reya, T. (2020). An in vivo genome-wide CRISPR screen identifies the RNA-binding protein Staufen2 as a key regulator of myeloid leukemia. Nature cancer, 1(4), 410-422.

[11] Quijada-Álamo, M., Hernández-Sánchez, M., Alonso-Pérez, V., Rodríguez-Vicente, A. E., García-Tuñón, I., Martín-Izquierdo, M., ... & Hernández-Rivas, J. M. (2020). CRISPR/Cas9-generated models uncover therapeutic vulnerabilities of del (11q) CLL cells to dual BCR and PARP inhibition. Leukemia, 34(6), 1599-1612.

[12] Sharma, A., Boelens, J. J., Cancio, M., Hankins, J. S., Bhad, P., Azizy, M., ... & LaBelle, J. L. (2023). CRISPR-Cas9 Editing of the HBG1 and HBG2 Promoters to Treat Sickle Cell Disease. New England Journal of Medicine, 389(9), 820-832.

[13] Xu, L., Wang, J., Liu, Y., Xie, L., Su, B., Mou, D., ... & Chen, H. (2019). CRISPR-edited stem cells in a patient with HIV and acute lymphocytic leukemia. New England Journal of Medicine, 381(13), 1240-1247.

[14] Fu, B., Liao, J., Chen, S., Li, W., Wang, Q., Hu, J., ... & Wu, Y. (2022). CRISPR–Cas9-mediated gene editing of the BCL11A enhancer for pediatric β0/β0 transfusion-dependent β-thalassemia. Nature Medicine, 28(8), 1573-1580.

[15] Gidding, S.S.; Allen, N.B. Cholesterol and atherosclerotic cardiovascular disease: A lifelong problem. J. Am. Heart Assoc. 2019, 8, e012924.

[16] Musunuru, K., Chadwick, A. C., Mizoguchi, T., Garcia, S. P., DeNizio, J. E., Reiss, C. W., ... & Kathiresan, S. (2021). In vivo CRISPR base editing of PCSK9 durably lowers cholesterol in primates. Nature, 593(7859), 429-434.

[17] Hu, Z., Wu, Y., Xiao, R., Zhao, J., Chen, Y., Wu, L., ... & Liang, D. (2023). Correction of F8 intron 1 inversion in hemophilia A patient-specific iPSCs by CRISPR/Cas9 mediated gene editing. Frontiers in Genetics, 14, 1115831.

[18] Tao, J., Bauer, D. E., & Chiarle, R. (2023). Assessing and advancing the safety of CRISPR-Cas tools: from DNA to RNA editing. Nature Communications, 14(1), 212.

[19] Chien, Y., Hsiao, Y. J., Chou, S. J., Lin, T. Y., Yarmishyn, A. A., Lai, W. Y., ... & Yang, Y. P. (2022). Nanoparticles-mediated CRISPR-Cas9 gene therapy in inherited retinal diseases: Applications, challenges, and emerging opportunities. Journal of Nanobiotechnology, 20(1), 511.

[20] Arai, D., & Nakao, Y. (2021). Efficient biallelic knock-in in mouse embryonic stem cells by in vivo-linearization of donor and transient inhibition of DNA polymerase θ/DNA-PK. Scientific reports, 11(1), 18132.

[21] Kim, H. K., Kim, Y., Lee, S., Min, S., Bae, J. Y., Choi, J. W., ... & Kim, H. H. (2019). SpCas9 activity prediction by DeepSpCas9, a deep learning–based model with high generalization performance. Science advances, 5(11), eaax9249.

[22] Cheng, X., Li, Z., Shan, R., Li, Z., Wang, S., Zhao, W., ... & Li, W. (2023). Modeling CRISPR-Cas13d on-target and off-target effects using machine learning approaches. Nature Communications, 14(1), 752.

[23] Saito, M., Xu, P., Faure, G., Maguire, S., Kannan, S., Altae-Tran, H., ... & Zhang, F. (2023). Fanzor is a eukaryotic programmable RNA-guided endonuclease. Nature, 620(7974), 660-668.