REVIEW PAPER

An Update on the Application of CRISPR Technology in Clinical Practice

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Abstract

The CRISPR/Cas system, an innovative gene-editing tool, is emerging as a promising technique for genome modifications. This straightforward technique was created based on the prokaryotic adaptive immune defense mechanism and employed in the studies on human diseases that proved enormous therapeutic potential. A genetically unique patient mutation in the process of gene therapy can be corrected by the CRISPR method to treat diseases that traditional methods were unable to cure. However, introduction of CRISPR/Cas9 into the clinic will be challenging because we still need to improve the technology's effectiveness, precision, and applications. In this review, we first describe the function and applications of the CRISPR–Cas9 system. We next delineate how this technology could be utilized for gene therapy of various human disorders, including cancer and infectious diseases and highlight the promising examples in the field. Finally, we document current challenges and the potential solutions to overcome these obstacles for the effective use of CRISPR–Cas9 in clinical practice.

Keywords CRISPR · Gene editing · Cancer · Gene therapy

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CRISPR Mechanism and its Various Types

Genome-editing technology is defined as sequence-based engineering for modifying DNA sequences and their variants, including insertions, deletions, integrations, and substitutions (Fig. 1). This targeted genome-editing technology is based on DNA repair mechanisms naturally present in cells [1, 2]. Moreover, site-specific genetic and epigenetic changes can be performed by combining programmable sequence-specific nucleases and regulatory proteins (Fig. 2) [3]. In recent years, CRISPR, which has naturally evolved as an immune system in many prokaryotes and archaea to defend against viral invasions, has been reprogrammed as an influential gene-editing tool that acts effectively in many organisms, including humans [4, 5, 6]. CRISPR technology uses CRISPR-associated (Cas) enzymes to cleavage specific nucleic acid sequences [7]. Because of its inherent programmability and simplicity, CRISPR technology is quickly replacing traditional gene-editing methods as the preferred tool for therapeutic gene editing. The CRISPR–Cas9 system has shown promise as a method for reversing gene alterations in diseases such as blood disorders and muscle degeneration as well as neurological, cardiovascular, renal, genetic, stem cell, and optical disorders. The CRISPR–Cas9
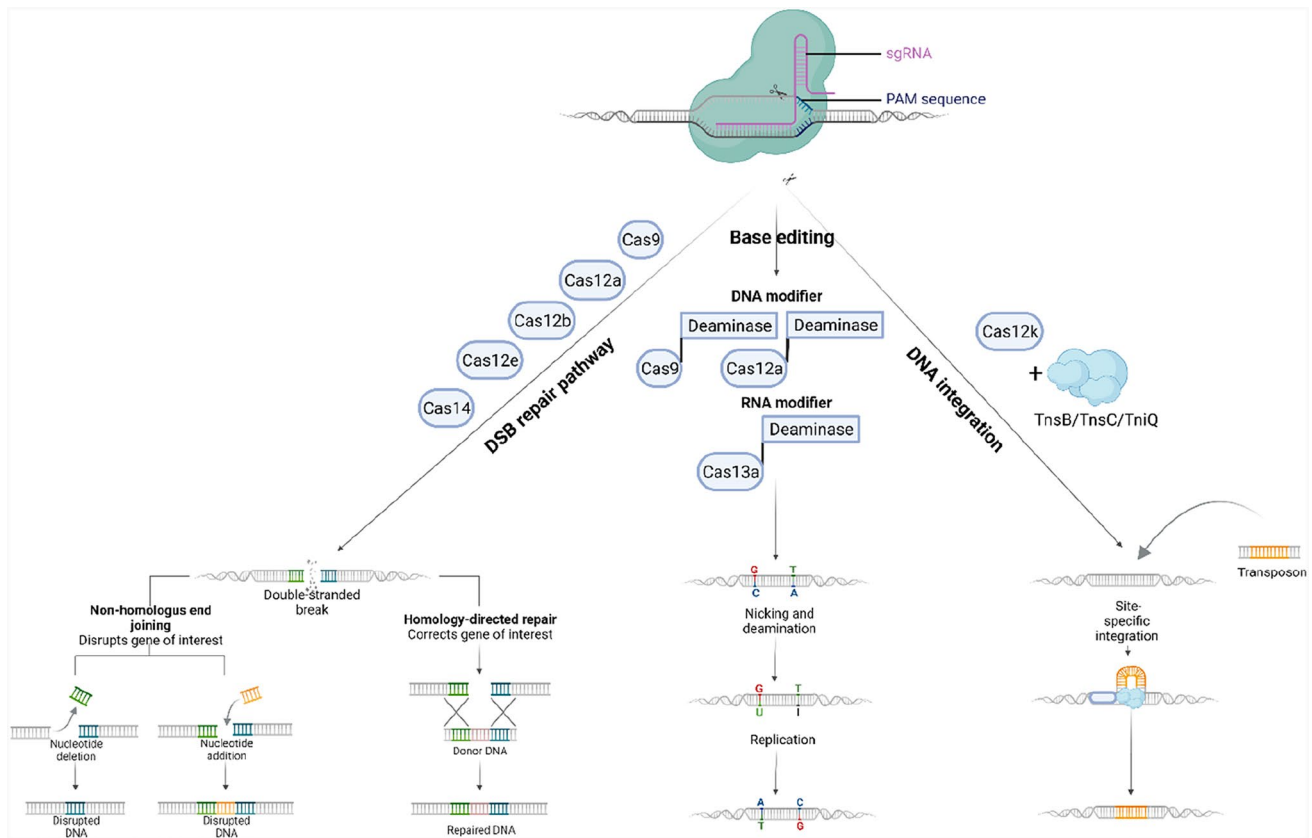
Fig. 1 Different types of genetic variants can be modified by Genome-editing technology. The figure shows the three mechanisms of variant repair, including double-strand break repair, base-editing repair, and DNA-integration repair, along with the involved factors that are used in genome-editing technology. Double-strand breaks are modified by two independent pathways including HDR and NHEJ. Substitution variants are modified by the base-editing mechanism. To integrate a specific DNA fragment to a specific site of genome, the DNA-integration repair mechanism is applied

system offers extraordinary therapeutic promise for treating a wide range of disorders when the genetic basis of the malfunction is understood, and it promotes better understanding of such disorders by developing numerous disease models. Gene function may be restored or a mutation can be compensated for by reprogramming treatments based on CRISPR–Cas9-mediated genome editing [8]. The CRISPR–Cas9 effector has been used in biosensing applications in addition to its impressive genome-editing capabilities. Certain CRISPR–Cas systems, such as orthologues of Cas13, Cas12a, and Cas14, have collateral non-specific catalytic activities that may be used for nucleic acid detection, through degradation of a tagged nucleic acid to create a fluorescence signal [9–11].

Several CRISPR-based gene-editing systems have been identified, each having specific enzymatic activity and nucleic acid binding requirements. However, most CRISPR applications have used Cas9 derived from Streptococcus pyogenes (spCas9) [7]. To target a specific DNA sequence, Cas9 employs a CRISPR RNA (crRNA) with a 20-nucleotide complementary sequence to the target sequence and a

trans-activating crRNA (tracrRNA) scaffold with a sequence recognized by Cas9 [12–14]. It should be noted that the two RNA fragments of CRISPR systems, crRNA and tracrRNA, can be integrated into a single-guide RNA (sgRNA) while maintaining the capacity for targeting and cleavage of specific nucleic acid targets sequences [15]. Unlike early gene-editing systems such as ZFN and TALEN, in CRISPR-based systems, targeting a new site at the genome level only requires changing the 20 nucleotides at the beginning of the sgRNA, which is involved in targeting Cas protein to the target sequence; this has made the transition between new gene targets much more efficient. Accordingly, CRISPR is rapidly transforming the state of research in the life sciences and medicine worldwide, moving toward clinical trials [7, 16, 17].

As mentioned above, several CRISPR–Cas systems are essential to be classified for a better understanding of the origin and also further research. The classification of the CRISPR systems is based on differences in the Cas protein compositions and the sequence differences between effector complexes. According to the classification suggested
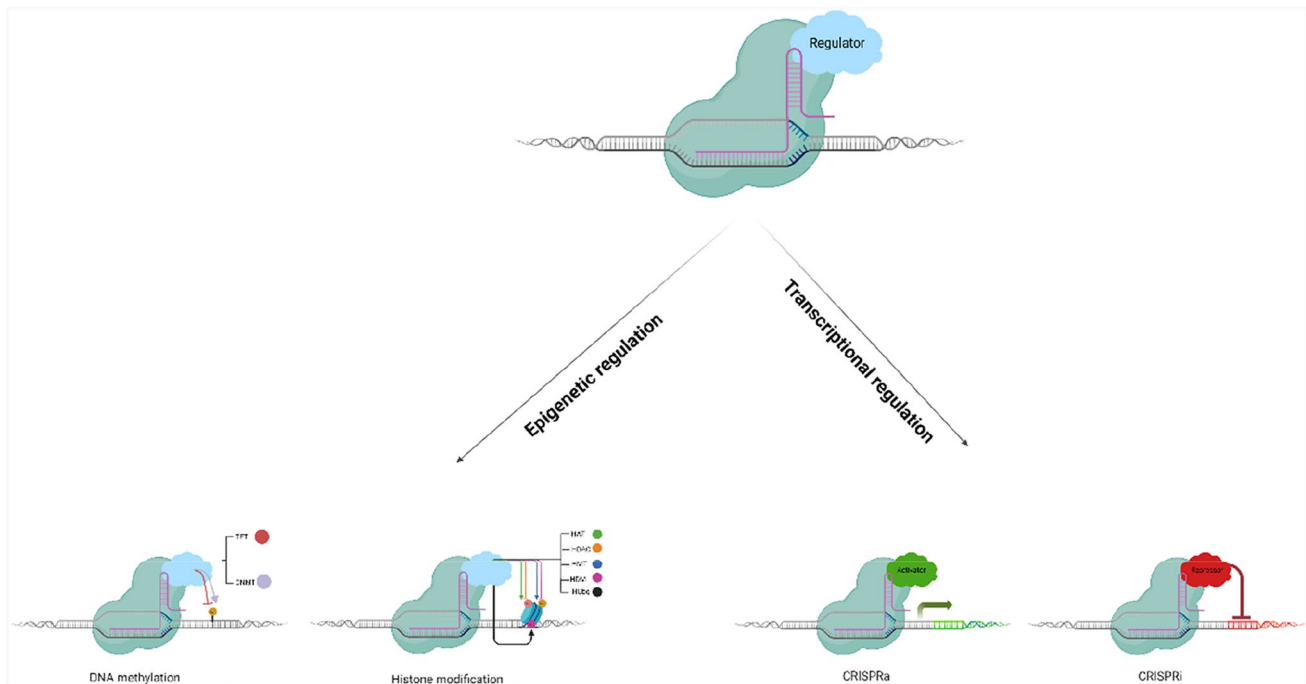
Fig. 2 A schematic scheme for epigenetic and transcriptional regulations made by combining programmable sequence-specific nucleases and regulatory proteins

by Makarova et al. in 2020, CRISPR systems are categorized into two classes, six types and 33 subtypes [18, 19]. Types I, III, and IV are included in the class I system, characterized by multiple subunit Cas proteins as effector complexes and a crRNA in the interference stage [18].

Type I consists of 7 subtypes: I-A, I-B, I-C, I-D, I-E, I-F, and I-G. The CRISPR-associated complex for an antiviral defense (Cascade) complex is considered an effector complex for the interference stage in type I systems. This Cascade complex typically comprises Cas3, Cas5, Cas7, Cas8, and other Cas proteins, depending on the subtypes. The Cas3, a crucial element of the Cascade complex in the type 1 systems, is necessary to cleave foreign DNA [20].

Type III has 6 subtypes: III-A, III-B, III-C, III-D, III-E, and III-F. Reverse transcriptase is present in some of the subtypes of type III in the adaptation module. The Cas6 proteins are applied to cleave pre-crRNA in the type III systems; however, most subtypes do not have any cas6 gene and resort to the Cas6 proteins supplied by other CRISPR–Cas loci. The subtypes III-A, III-D, III-E, and III-F, utilize a Csm complex consisting of Csm/Cas proteins and crRNA, and subtypes III-B and III-C profit from a Cmr composed of Cmr/Cas proteins and crRNA as effector complex. Different subtypes have different targets for cleavage by their effector complex; in subtypes III-A, III-B, and III-C the target is DNA/RNA, in subtypes III-D and III-E, it seems to be RNA, and in subtype III-F it is expected to be DNA [18, 20–22].

Type IV consists of 3 subtypes: IV-A, IV-B, and IV-C. The Cas proteins are not usually present in these systems in the adaptation stage and the cleavage of foreign targets. The cleavage of pre-crRNA is usually performed using a specific Cas6 protein. It has been suggested that Cas5, Cas7, and the large subunit (Csf1) proteins comprise effector complexes of type IV systems. With regard to the class I CRISPR systems, because of insufficient knowledge and difficulties in cloning the multiple effector complexes in a functional vector or its production in the form of ribonucleoprotein (RNP) complex, its routine use as a genome-editing tool is limited. Therefore, the class II systems that had the advantage of inducing various genetic modifications were considered for genome editing [18, 23–25]. In contrast to the class I systems, the class II systems have a single and large multidomain effector complex linked to a crRNA, subclassified into three types: type II, type V, and type VI.

Type II contains three subtypes, including II-A, II-B, and II-C. Three components are necessary for pre-crRNA processing in type II systems: RNase III, which is used for the maturation of pre-crRNA, the Cas9 protein that identifies the PAM sequence and is directed to one strand of the DNA target, and tracrRNA, which is required for target recognition. The processing of pre-crRNA in type II-C differs from other subtypes of the Type II systems [18, 20, 26–33].

The Type V systems are composed of 10 subtypes: V–A, V–B, V–C, V–D, V–E, V–F, V–G, V–H, V–I, and V–K, utilized from the Cas12 protein as a single effector

complex. Processing of pre-crRNA varies in the different subtypes of Type V systems by applying the effector complex in subtype V-A and RNase III in several other subtypes. Unlike the Type II systems, double-strand cleavage of DNA target is performed by Cas12 protein [18, 34–46].

The Type VI systems consist of 4 subtypes: VI-A, VI-B, VI-C, and VI-D. The single effector complex used in Type VI is the Cas13 protein, which differs from the other effector complexes in the class II systems. The effector complex is responsible for the processing of the pre-crRNA. The effector complexes involved in Type VI have two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains that cooperate in the RNase activity. Instead of the PAM required for double-strand

DNA cleavage, these systems need a protospacer flanking sequence (PFS) to cleave ssRNA targets [47–50] (Table 1).

Clinical Applications of the CRISPR System

Cancer Treatments

Cancer is a complex disorder caused by genetic and epigenetic mutations in oncogenes and tumor suppressor genes. CRISPR–Cas9 can be used to manipulate the genome in tumor research and to investigate the causes of tumor initiation, progression, and metastasis. This system has been widely used in cancer treatment investigations in recent

Table 1 Different types of the CRISPR–Cas systems [18, 24]

Class	Type	Subtypes	Target	Spacer integration	Pre-crRNA processing	Effector complex	Target cleavage
I	I	I-A	DNA	Cas1, Cas2, [Cas 4]	Cas6	Cas7, Cas5, SS*, Cas8/LS	Cas3", Cas3'
		I-B	DNA				
		I-C	DNA				
		I-D	DNA				
		I-E	DNA				
		I-F	DNA				
		I-G	DNA				
	III	III-A	DNA+RNA	Cas1, Cas2, [RT]	[Cas6]	Cas7, Cas5, SS, Cas10/LS	Cas10/LS
		III-B	DNA+RNA				
		III-C	DNA+RNA				
		III-D	RNA?				
		III-E	RNA?				
		III-F	DNA?				
	IV	IV-A	unknown	[Cas1], [Cas2]	[Cas6]	Cas7, Cas5, [SS], Csf1/LS	unknown
		IV-B	unknown				
		IV-C	DNA?				
II	II	II-A	DNA	Cas1, Cas2, [Cas 4]	RNase III	Cas9	Cas9
		II-B	DNA				
		II-C	DNA				
	V	V-A	DNA	[Cas1], [Cas2], [Cas 4]	Cas12	Cas12	Cas12
		V-B	DNA				
		V-C	DNA				
		V-D	DNA				
		V-E	DNA				
		V-F	DNA				
		V-G	RNA				
		V-H	unknown				
		V-I	DNA				
		V-K	unknown				
	VI	VI-A	RNA	Cas13	Cas13	Cas13	Cas13
		VI-B	RNA				
		VI-C	RNA?				
		VI-D	RNA				

years by correcting mutations or knocking out certain genes [51]. Numerous exploratory investigations on tumor treatment in related disciplines have been undertaken to date. The CRISPR–Cas9 applications are now mostly focused on single-gene disorders, viral infections, and cancers [52]. CRISPR-based therapies are currently used to treat blood cancers such as leukemia and lymphoma. In addition, a lung cancer trial in China was recently completed [53]. In 2016, CRISPR therapy was used in a lung cancer patient. This patient was injected with PD-1 altered T cells. Another clinical trial in the United States using CRISPR-based cancer immunotherapies has also been completed [54]. Numerous other clinical trials using CRISPR-based immunotherapies are ongoing for treatment of cancers. In a Chinese human trial, researchers from the Sichuan University's West China Hospital used PD-1-modified T cells to treat 12 patients with non-small-cell lung cancer. CAR-T was not included in this strategy since it is not currently available for lung cancer. The study's major objectives were to see if the treatment was safe, had manageable side effects, and evoked a positive response[55]. The other completed study was the safety evaluation of the PD-1 knock-out engineered T cells in treating advanced esophageal cancer, conducted in China (5). In addition, several studies are recruiting for treatments of solid tumors, gastric carcinoma, nasopharyngeal carcinoma, hepatocellular carcinoma, renal cell carcinoma, B cell leukemia, and lymphoma.

Infectious Diseases

To date, infectious diseases are one of the most common global causes of serious and lethal human disorders [56]. These diseases are the cause of frequent epidemics and demand preventive care and are sometimes difficult to treat. In addition, many of them are resistant to antibiotics or have vaccine-escape mutations; unsurprisingly, in this case, latent infections are expected [57]. Viral and bacterial pathogens are major global healthcare challenges. In the last two decades, the WHO introduced a number of infectious diseases (H1N1 Influenza, Polio, Ebola, Zika, Kivu Ebola, and COVID-19) as "public health emergency of international concern". In addition, other pathogens like HBV, HCV, HIV, HPV, HSV, mycobacterium tuberculosis is threatening public health worldwide [57].

Infectious diseases can be divided into two groups: 1. Bacterial infections, and 2. Viral infections. Bacterial infection treatments are mainly based on antibiotic drugs. However, due to overuse of antibiotics, inappropriate use of antibiotics, and their presence in feedstock, enabling bacteria to adapt and develop continually against the medications, the risk of multidrug-resistant (MDR) bacteria is growing as a major challenge. Additionally, the developed resistance is easily transmittable from one host to another. However, there is a hope that this threat can be better addressed with CRISPR–Cas9, emerging as a major tool that targets the genome of resistant strains in a sequence-specific manner [58].

COVID-19

The recent COVID-19 pandemic caused by Severe Acute Respiratory Syndrome Coronavirus-2 (also known as nCoV-2 or SARS-CoV-2) has become an important global health problem in the last three years because it is responsible for more than 6.5 million (https://www.worldometers.info/coronavirus) deaths worldwide and enormous economic and social limitations [59]. However, the antiviral drug Veklury (remdesivir) has been approved for the treatment of patients (Oct 2020); Some others, such as monoclonal antibody therapies, have been approved through the emergency use authorization (EUA) [60]. Despite the short time since the emergence of COVID-19, the virus was widely targeted in clinical trials and several distinct types of vaccines have been developed [60]. Today, many clinical trials are registered in different databases with the keyword of COVID-19, but a few are associated with the CRISPR technology. These trials can be divided into two groups: −1. Diagnostics (in vitro), −2. Cell therapies (ex vivo).

Because of CRISPR's high specificity and sensitivity [56, 61], among other diagnostic methods such as RT-PCR, immunoassay, isothermal nucleic acid amplification technique (iNAAT), and biosensors, this technique was immediately used in diagnostics as a point-of-care test [59]. CRISPR-facilitated detection could be a powerful screening and genotyping method for COVID-19 infection. The CRISPR/Cas technology has been widely used in clinical trials to provide a CRISPR-based Point-of-Care (POC) test. There are multiple registered clinical trials such as chictr2000029810 (2020), NCT05034978 (2021), and NCT05107258 (2021), aiming to improve the sensitivity and specificity and reduce the time, cost, and required instruments. The first FDA-Approved (May 2020) CRISPR/Cas tool to diagnose SARS-CoV-2 was Sherlock™ with 100% sensitivity and specificity in an EUA clinical evaluation (https://sherlock.bio/crispr-sars-cov-2/). Another two methods namely DETECTRTM in Aug 2020 and DETECTR BOOST (https://mammoth.bio/covid/) in Jan 2022 were introduced. These tools used CRISPR/Cas13 or CRISPR/Cas12 to detect specific sequences in the COVID-19 genome. However, studies that have used the CRISPR technique to diagnose COVID-19, are not limited to those mentioned above. The CRISPR technology has not only been used to detect COVID-19 but some studies have improved its diagnostics capacity by combining CRISPR with other techniques such as LAMP, biosensors, microfluidics, and nanoparticles [62]. Interestingly,

the detection limit has been increased up to < 20 copies/sample LOD and reached 100% sensitivity and specificity that could be performed in 20–40 min [59].

In addition, some trials have focused on engineered cell therapies using the CRISPR/Cas technology. It has been shown that the severity of COVID-19 could be changed by modifying the immune system [63, 64]. Thus, some scientists have focused on autologous T-cell therapies to treat the infection. Various cell types including CAR-T and CAR-NK, can be successfully modified today via CRISPR/Cas strategies. PD-1 knocking-down is a well-known strategy to circumvent the inhibitory immune system checkpoint [65, 66]. In this method, exhausted virus-reactive CD8 + memory T cells are isolated from patients. The PD-1 and/or ACE2 genes are then knock-out via the CRISPR/Cas technique to provide modified memory T cells and induce long-term immunity against COVID-19. The engineered lymphocytes are expanded "ex vivo" and infused back into patients. The PD-1 and/or ACE2 knock-out T cells have been tested in a trial by the Kafrelsheikh University in 2021 (NCT04990557). The result of this study has not yet been published.

The weakness of the immune or cell therapy is the efficiency reduction against new variants with significant mutations in surface proteins. This weakness could be obviated by direct targeting the conserved sequences in viral RNA. The CRISPR system has been demonstrated to degrade the viral RNA directly and terminate the infection; thus, as observed in recent studies it could result in up to > 5000-fold reduction in viral titer, which is more effective than antiviral drugs or vaccines [67]. However, the data supporting the advantages of targeting the viral genome are limited to preclinical studies, and no clinical trials have been registered so far.

In the case of COVID-19 and other similar infectious diseases, due to the high transmissibility and severe pathogenicity and the possibility of the formation of diverse mutant strains, early diagnosis and/or treatment of the disease is very important; thus, the CRISPR technology with the ability to be applied in POC tests as well as improving the function of the immune system by targeting the viral RNA has a great potential to applied in such pandemics as one of the first options in the process of diagnosis and treatment.

Human Immunodeficiency Virus (HIV)

HIV/AIDS is one of the fatal diseases that has infected 39 million people worldwide. Even though life-long anti-retroviral therapy can take HIV replication under control in a "shock-and-kill" manner [68], the persistence of HIV proviral, known as latent infection, is a barrier to HIV cure. During latency, HIV reduces the expression of viral proteins, which precludes the immune system from arising the infection. Thus, eliminating HIV DNA from infected individuals

is still the biggest challenge in HIV treatment. There are two strategies for HIV treatment, both of which are based on gene therapy strategies: targeting the provirus [68, 69] and targeting host genes that are crucial for the entrance of viruses into cells (CCR5 [70] and CXCR4 [71]). Nowadays, these approaches are achieved by CRISPR/Cas techniques.

HIV was the first infectious disease targeted by gene therapy in a clinical trial [72]. It has been shown that CCR5-null blood cells are resistant to HIV-1 entry, and this raises the possibility that CCR5 knocking out via CRISPR/Cas may be an alternative approach to clear HIV provirus from the cells. In 2017, in a trial (NCT03164135), CRISPR/Cas9 (with a non-viral delivery system) was successfully employed to induce indels (insertion or deletion mutation in genome) in the CCR5 gene in donor-derived HSPCs (CD34 + cells). Although a 19-month follow-up showed that the CCR5 knock out efficiency was only 5.2—8.28% in the bone marrow, it has been reported that no off-target effect occurred [73]. Thus, engineered HSPCs and allogeneic transplantation in a patient with HIV infection could be considered a potential therapeutic strategy for HIV infection and acute lymphoblastic leukemia. In addition, the Excision BioTherapeutics announced a phase 1/2 trial (NCT05144386 & NCT05143307, 2021) to study the efficiency of a single IV dose of a CRISPR/Cas9, which is delivered via an AAV9 delivery system, called "EBT-101," to HIV-infected adults. The study is recruiting participants.

Surprisingly, the world's first genome-edited babies (twin girls) were born in 2018. The study was done by the Southern University of Science and Technology of China in Shenzhen to impregnate a woman with genome-edited embryos in which the pathway that HIV uses to infect cells were genetically disabled. Researchers used the CRISPR/Cas9 system to destroy the CCR5 gene in the human germline [74, 75].

Human Papilloma Virus (HPV)

Human papilloma virus—HPV is the main cause of cervical intraepithelial neoplasia (CIN) and cervical cancer [57]. Evidence suggests that CRISPR-based strategies have a great potential to target HPV-related malignancies. Since E6 and E7 genes (major oncogenes) have important roles in virus replication, life cycle, and HPV-derived carcinogenesis, by affecting the P53 and RB pathways, respectively, they have been attractive therapeutic targets [76–78]. Repression of E6 and E7 genes significantly accumulates p53 and p21 and induces apoptosis and inhibits cell growth [57, 79]. Soon after that Yu and colleagues (2014) for the first time targeted HPV16 E6 by CRISPR [80], the phase 1 trial (NCT03057912, 2017) was designed to assess the safety and therapeutic doses of the CRISPR/Cas9 system to treat human cervical neoplasm and HPV persistency by the First Affiliated Hospital, Sun Yat-Sen University. This trial used

the E6 and E7 genes in HPV genome as the CRISPR/Cas9 targets. Unfortunately, there is no published data to report the study results.

HPV is an important human pathogen and its treatment was the subject of many studies and trials as a result of its transmission manner, prevalence rate, and malignant outcomes. Even though there is vaccination against HPV, there is no treatment for this virus [76]. As with the different variants of COVID-19, the presence of 150 different types of HPV can be a problem for the effectiveness of routine vaccines and treatments. Since it was observed that targeting E6/7 results in the accumulation of p53 and p21, and that it was suggested that HPV16 E6/E7 inhibition by CRISPR could be a sensitizer for CDDP chemotherapy [79–83]. Research teams have increasingly hoped to treat HPV-related malignancies, by the CRISPR technique.

Based on ongoing studies, Double-stranded DNA (and RNA genome) can be sensed and degraded effectively by the CRISPR system; Identification of new Cas variants and sgRNA-free systems allows scientists and clinicians to use the ultra-sensitivity and specificity of this technique in the diagnosis and treatment of infectious diseases.

Blood Disorders

Sickle cell disease (SCD) and transfusion-dependent β-thalassemia (TDT) are the most common monogenic disorders. About 300,000 patients with SCD and 60,000 patients with TDT are diagnosed each year[84–86]. These hemoglobinopathies resulted from hemoglobin subunit β (HBB) gene mutations. It has been reported that more than 200 mutations can occur in the β-globin gene [87], including single-base substitutions, Mutations that lead to small insertions or deletions within the gene, mutations affecting the transcription process, RNA translation, or RNA processing. Mutations inhibiting translation and more infrequently, deletions of the open reading frame [88], or deletions of a large part of the regulatory sequences in the locus control region (LCR) lead to either a partial deficiency (β+-thalassemia) or lack (β0-thalassemia) of β chains and an imbalance between β-like and α-like (e.g., β, γ, and δ) hemoglobin chains. Accumulating additional α-globin chains in red blood cell precursors leads to cellular damage, a process that results in ineffective maturation of erythroid, defective erythropoiesis, and reduced survival in red blood cells (RBC) [89]. A combination of unsuccessful erythropoiesis and shortened survival of RBC causes anemia. In the absence of appropriate treatment, marrow cavities expansion and enormous extramedullary cell proliferation lead to hepatosplenomegaly, skeletal deformity, and extramedullary pseudotumors [90]. The current therapeutic approaches for β-thalassemia

consist of regular transfusion combined with an iron chelator agent, splenectomy, hematopoietic stem cell (HSC) transplantation, and gene and cell therapy [91, 92]. SCD results from a mismatch mutation in HBB that leads to glutamic acid to valine substitution at the codon six position. Deoxygenated sickle hemoglobin polymerization results in deformation and hemolysis of erythrocyte, anemia, organ damage, vaso-occlusive crisis, and reduction of life span. Curative approaches for SCD include pain management, transfusion, hydroxyurea, hematopoietic stem cell (HSC) transplantation, and gene and cell therapy. Allogeneic bone marrow transplantation (BMT) or HSC transplantation from appropriate donors provided promises for curing both SCD and TDT. However, lack of compatible donors for a high proportion of patients, Graft versus host disease (GVHD), and graft failure limit the application of this strategy. Disadvantageous of allogenic BMT may be resolved by using autologous gene-corrected HSCs [93].

The HBB gene mutations can be edited with strategies such as CRISPR/Cas9 [94, 95]. There are two main approaches for curing TDT and SCD by the CRISPR/Cas9 system [96]. One approach focuses on the correction of causative mutations. The other approach is γ-globin reactivation, compensating for the β-globin deficiency. EIF2AK1 is a heme-regulated inhibitor (HRI) and a red blood cell-determined kinase that interferes with the translation of HbF. In addition, BCL11A is a suppressor of HbF. Down-regulation of BCL11A results in a low level of HRI production which eventually increases the HbF production [97]. Different molecular analyses have shown that inhibition of BCL11A results in HbF overexpression [98]. CRISPR/Cas9 has been utilized to restore the γ-globin-associated genes through inactivation of their repressor, BCL11A, or inhibition of BCL11A binding to its target site. Therefore, this strategy leads to γ-globin overexpression and diminishes the severity of β-thalassemia symptoms [99]. A phase 1/ 2/3 clinical trial (NCT03655678) used this approach for β-thalassemia treatment. Briefly, a TDT patient was administered with CTX001 (autologous CD34 + HSPCs that were genetically modified using the CRISPR/Cas9 system to induce fetal hemoglobin production through down-regulation of BCL11A). In a similar clinical trial (NCT03745287), CTX001 was infused into an SCD patient [100]. The results of these trials have been published recently [101]. After infusion of CTX001, both patients had significant and constant rises in fetal hemoglobin levels. Higher levels of fetal hemoglobin (containing two gamma and two alpha chains) ameliorated complications and mortality in patients with TDT and SCD [102–104].

In addition, a long-term follow-up project for patients that received CTX001 has been started (NCT04208529). Another clinical trial (NCT03728322) has been initiated to study the combination of autologous, iPSC-derived HSCs with a

direct edition of the HBB gene in β-thalassemia. However, the exact approach of the trial is not determined. In a phase 1 clinical trial (NCT04925206), autologous CD34 + human hematopoietic stem and progenitor cells (hHSPCs) were gene-corrected using the CRISPR/Cas9 system and administrated in a single dose to TDT patients. In another phase 1/2 clinical trial (NCT04819841), an autologous gene-corrected CD34 + HSCs (HbS to HbA) called GPH101 was used to cure severe SCD. There is another phase 1/2 clinical trial (NCT04774536) for treating severe SCD patients using the CRISPR/Cas9 approach (CRISPR-SCD001). However, more information on this study was not provided.

Neuroscience

Nerves system function depends on a balanced gene expression, especially in the Neuroglia cells (Also called glial cells: astrocytes, oligodendrocytes, and microglia); however, this balance is commonly disrupted in manifested neurodevelopmental, neuropsychiatric, and neurological diseases [105]. In neurodegenerative and neurodevelopmental disorders such as Alzheimer's disease—AD, Huntington's disease—HD, and Parkinson's disease—PD, progressive neuronal dysfunction is mainly caused by dysregulation of the neuron–glia and glia–glia networks [105]. On the other hand, in Neuromuscular Disorders, the peripheral nervous system and the muscles are affected [106]. Thus, Neuromuscular disorders cause significant incapacity, even almost complete paralysis. This category of disorders consists some famous diseases as well as amyotrophic lateral sclerosis (ALS), Duchenne muscular dystrophy (DMD), and spinal muscular atrophy (SMA). It should be noted that both neurodegenerative and neuromuscular disorders are a heterogeneous class of diseases [106]. In fact, there are 16 different groups of neuromuscular disorders with so many illnesses in each group (http://www.musclegenetable.org/). In addition to adverse effects of these disorders on the quality of life, these conditions impose an extensive financial burden on society. Unfortunately, the existing pharmacological treatment options provide only transitory symptomatic relief without influencing the underlying disease causes. Therefore, "Gene Therapy" has been used for Neurological, Neurodegenerative, and Neuromuscular disorders and is currently being applied by novel gene-editing systems like CRISPR/Cas techniques. Although CRISPR sequences were identified in 1978 [107] and introduced as a powerful genome-editing system in human cells by 2013 [57], immediately in 2016, it was utilized in a clinical trial (NCT02793856) [108, 109]. In addition to its huge therapeutic prospective for human genomic DNA editing [105, 109], the CRISPR–Cas9 system has been used to target human genetic disorders in vivo and in vitro systems. Numerous preclinical studies have shown the benefits of CRISPR/Cas9-mediated gene editing

for the treatment of both neurodegenerative and neuromuscular disorders. Many studies utilized CRISPR strategies to ignore the premature stop codon in the DMD gene (the cause of Duchenne muscular dystrophy) by creating indels in adjacent exons [66, 110], which led to recovery of dystrophin expression [111]. Recently, engineered Cas9 proteins, termed "Base Editors" and "Prime Editors," were used to induce exon skipping by altering sequences in splice site [112, 113]. Moreover, it can be used to correct the point mutations which are persisting in 25–35% of DMD patients. It has been demonstrated that approximately 20% gene-editing efficiency could be sufficient to restore wild-type protein levels and deminish disease symptoms [113, 114]. The SOD1, C9orf72, ATXN2, and FUS genes are associated with Amyotrophic lateral sclerosis (ALS). Since 2017, CRISPR systems have been used to disrupt SOD1 gene expression [115–117]. Recently, a study was done to generate indels in iPSCs derived from a patient with C9orf72 expansions [118]. Spinal muscular atrophy (SMA) is a fatal neuromuscular disorder resultig from the loss-of-function mutations in the survival motor neuron 1 (SMN) gene; thus, gene therapy aims to restore the SMA gene's functions. A study successfully increased the production of SMN2 full-length (FL) gene expression by a CRISPR/Case 9 targeted intronic-splicing silencer sequence [119]. Accumulation of amyloid-beta (Aβ) and tau proteins are related to alzheimer's disease—AD pathophysiology. Since many genetic factors such as oxidative stress, vascular dysfunction, proteinopathy, metal ions, inflammation, mitochondrial dysfunction, lipid metabolism, microbiota–gut–brain axis, and gene-environment interactions are associated with the pathophysiology of AD [125], various studies proposed either knocking out APP, PSEN1, PSEN2, CysLT1R, GMF, BACE1, Th genes [120–123] or correcting APOE4 alleles to E3 or E2 as a potential treatment approach for AD [121, 124]. Various CRISPR/Cas systems were used as powerful techniques to reach this goal. CRISPR/Cas-based treatments are applied to solve the consequential neuronal toxicity in the huntington's disease—HD patient's brain, mainly by targeting the HTT gene [125–129] or mRNA [130]. These approaches can achieve up to a 79% reduction in HTT production [130]. Toxicity in Parkinson's Disease—PD is caused by an increase in the kinase activity of LRRK2 and Lewy bodies (LBs) appearance; thus, gene-editing systems could be successfully used to treat PD molecular features by targeting the SNCA (α-synuclein), LRRK2, PARKIN, and INK1 genes especially using the CRISPR/Cas system [131, 132]. Notwithstanding, so studies have been registered on the topic of neurodegenerative, neurodevelopmental, and neuromuscular disorders; there are a few registered clinical trials using gene therapy techniques, and unfortunately, very few clinical trials have investigated the CRISPR/Cas method. The CRISPR/Cas system has recently been employed to

treat genetic disorders in vivo. The first-ever clinical trial reported clinical safety and molecular efficacy of an intravenous (IV) administration of the CRISPR/Cas system in vivo in human-targeted ATTR. This clinical trial (https://ir.intel liatx.com/news-releases/news-release-details/intellia-thera peutics-doses-first-patient-landmark-crisprcas9), Supported by Intellia Therapeutics and Regeneron Pharmaceuticals. (NCT04601051), designed (in New Zealand and the United Kingdom, 2020) to elevate the safety, tolerability, pharmacokinetics, and pharmacodynamics of a single dosage infusion therapy against Hereditary Transthyretin Amyloidosis with Polyneuropathy (ATTRv-PN) and Transthyretin Amyloidosis-Related Cardiomyopathy (ATTR-CM). This therapy, named "NTLA-2001," is based on the CRISPR/Cas strategy and uses a lipid nanoparticle delivery system to target the TTR gene in hepatocytes. In an ongoing phase 1 clinical study, a single dose of NTLA-2001 was tested in six patients diagnosed with hereditary ATTR amyloidosis with polyneuropathy (three patients were diagnosed with the p.T80A mutation, two had the p.S97Y mutation, and one had the p.H110D mutation). The results showed (after 28 days after treatment) [133] a durable knock-out of the TTR gene in a dose-dependent manner and a 47% to 96% reduction in serum TTR protein level correlating with dosage. This level of response, besides a few adverse events observed in this trial, could be scaled satisfactorily for the treatment to be offered to all ATTR patients [134].

Another clinical trial (NTC03332030) with 20 enrollments has been done in the field of Neurosciences, which is supported by the Children's National Medical Center in the United States (2015). This trial focused on Optic pathway gliomas—OPG, a low-grade astrocytic tumor primarily involving the optic nerve, chiasm, and tracts that occurs in 20% of children with NF1. However, low-grade astrocytic gliomas formation in the other parts of the brain, such as the hypothalamus, brainstem, and cerebellum are also usual. These brain tumors can grow at diverse rates and may lead to neurologic dysfunction ranging from severe disabilities to little or no symptomology. Thus, this trial aimed to achieve three different engineered nervous system cell lines (NF1 wild-type NF1 + / + , NF1 Heterozygous NF1 ± , and NF1 Homozygous NF1 -/-) that were differentiated from the induced pluripotent stem cell or iPSCs.

Nowadays, the entire genome can be targeted by CRISPR, either by degrading or by base-editing. Among neuroscientists, CRISPR has been well accepted because this method is completely compatible with scientific purposes and provides a provision for conducting preclinical and clinical practices. It is expected that CRISPR–Cas will pave the way for the operationalization of neuroscience processes, by facilitating the production of the required animal models and cell lines of neurological diseases to accede a better understanding of the disease mechanism and to apply the

existing gene therapies and develop the translational neuroscience. However, it should be noted that at present two weaknesses hinder the clinical application of CRISPR in genetic diseases related to the nervous system. Therefore, the delivery methods as well as minimizing the off-target effects of the CRISPR–Cas is the first step that should be improved, thereby CRISPR can promote gene therapy of neurological diseases. Notwithstanding, these issues do not detract from the immense value and potential of the CRISPR–Cas technique.

Periodontic Disorders

Periodontitis is a serious gum disorder with inflammation and infection that affects the soft connective tissue and can damage bone tissue if left untreated [135]. The CRISPR technology can be used to target microbes associated with oral diseases. For example, bacteriophage engineering through the CRISPR–Cas3 system can target oral pathogens and biofilms [136]. CRISPRa, CRISPRi, and Cas13 systems also knock down the genes involved in periodontal disorder by altering the transcriptome without changing DNA sequences. The advantage of this method over traditional DNA editing is that the changes are reversible and temporary [134]. By knocking out a gene using the CRISPR technique, its role in biological pathways can be understood. It was documented that knocking out the PTPN2 gene with CRISPR–Cas9 in mouse models enhances the phosphorylation of JAK1 and STAT3,22 transcription factors, leading to increased periodontal inflammation. Therefore, it can be concluded that PTPN2 contributed to the inhibition of this inflammation [137–139]. Overall, the usage of the CRISPR method in periodontitis experimental trials has been limited; however, with the development of this system, more therapeutic strategies will replace surgery to treat periodontal disorders.

Rare Protein Folding Diseases

One of the rare proteins folding diseases is Hereditary transthyretin-derived amyloidosis (ATTRm) which has a prevalence of fewer than 1/100,000 people worldwide and is associated with sensory and motor neuropathy [140–142]. ATTR is similar to other neurological diseases caused by protein misfolding, such as Alzheimer's and Parkinson's. In this disorder, the mutant TTR protein is incorrectly folded, sticks together to form fibril amyloid plaques, and even interferes with the function of normal TTR proteins. Recently, it has been shown that the CRISPR technique can play a promising role in treating and managing ATTR by modifying the gene involved in this rare disorder. The first CRISPR–Cas9 clinical trial, in which genome-editing components were

systematically delivered to the body by lipid nanoparticles, was used in the treatment of ATTR. In this method, the NTLA-2001 genome-editing agent based on CRISPR–Cas9 is used to create a double-strand break in the TTR gene, and thus decrease the production of the defective protein and its aggregation. In fact, a 52% reduction in serum TTR protein levels was found after 28 days. This in vivo CRISPR therapy was started in the United Kingdom in 2020 and is currently recruiting; this project is funded by Intellia Therapeutics in conjunction with Regeneron Pharmaceuticals [133].

Preimplantation Genetic Diagnosis

CRISPR is introduced as a therapeutic tool for modifying genetic disorders in early embryos or germline cells. At the same time, Preimplantation genetic diagnosis (PGD) is a selective method for finding embryos that do not have genetic abnormalities [143]. However, PGD is not always effective, for example, in cases where both parents are heterozygous for an autosomal dominant disease or when the mother is heteroplasmic for a mitochondrial mutation, it is less likely to find enough embryos without harmful mutations. Besides, if one parent is homozygous for an autosomal dominant condition, both are homozygous for an autosomal recessive disease, or the mother has a homoplasmic mitochondrial mutation, it is not possible to transfer healthy embryos by the PGD method because all of them are affected by inherited mutations. In this situation, the CRISPR technique can be used to have healthy offspring [144–146]. Clinical application of the CRISPR system on human embryos is in the early stages, and limited studies have been conducted in this regard. The efficiency of Zygote's genetic modification by CRISPR–Cas9 has been reported to be between 0.5 and 40%. So far, CRISPR germline genome editing (CRISPR GGE) has been used in model animals (like primates and mice) to correct genetic errors in diseases such as beta-thalassemia. It has also been reported that CRISPR GGE can prevent SMA births in mice [147]. Today, there are concerns about the safety of CRISPR applications in reducing abnormal genetic births and altering the human genetic pool. However, this technology will be a potential prenatal treatment approach in the future [143].

Mitochondrial Disorders

Mitochondrial disorders, also called "Mystondria" (mysterious diseases of mitochondria), are a group of inherited diseases that occur due to mutations in mitochondrial DNA—mtDNA or in nuclear DNA—nDNA. The prevalence of these disorders is increasing today, and there is no therapeutic application to treat mitochondrial disease. Therefore, genome-editing techniques, especially the CRISPR/Cas System, were widely used to treat these diseases by

manipulating the nDNA or targeting the mtDNA [148]. On the one hand, special features of mtDNA make it necessary to apply specific genome-editing methods. On the other hand, Cas9 is a large protein; likewise, RNA importing processes to mitochondria is unclear. Therefore, delivering the CRISPR/Cas9 package into the mitochondria is challenging. Thus, more studies are needed to validate the efficiency of the CRISPR/Cas9 system in mitochondria-associated disorders treatment. From the future perspective, identifying or engineering new Cas proteins, eliminating the need for the guide RNA, and designing new methods to deliver the CRISPR system into the mitochondria of the target cells are the main goals that must be achieved first in order to enhance the gene-editing potential of the CRISPR method for mtDNA.

The history of applying the CRISPR/Cas system to mitochondrial disorders dates back to 2015. Initially, to study mitochondrial diseases, cell lines and animal models of the disease were required, which were developed by genome-editing techniques such as CRISPR/Cas9 (e.g. NSUN2 knocked-out HEK293T cell line [149–151], *YARS2* knocked-out HeLa cell line [152], and *yars2*$^{-/-}$ zebrafish, *nsun2*$^{-/-}$ mice [150] model). At the same time, the CRISPR technique is used in the development of Genomic-Wide Screening Libraries to identify essential genes in different pathways such as oxidative phosphorylation [153], ATP-modulating [154], cell death [155], metabolic resistance [156], adenine nucleotide translocator—ANT functions [157], or knock-out screen to identify how mitochondrial stress is relayed to ATF4 [158].

Despite the colossal number of studies that have introduced the clinical applications of CRISPR/Cas systems, its use has limitations for mitochondrial disorders because the delivery of the CRISPR system into mitochondria remains debatable. In 2015, the nuclear localization signal on the N-terminus of the Cas9 protein was replaced with a mitochondrial targeting sequence (MTS), called "mitoCas9," [159] to target the MT-COX1 and MT-COX3 genes in the HEK293T cell line. After five days, a significant decrease in mtDNA was observed. Subsequently, many studies have been performed to improve the CRISPR system or its delivery method to mitochondria [159–161].

Challenges of gene editing by the CRISPR system

CRISPR/Cas9 is a promising method, but its recent discovery and application in humans makes it difficult to utilize in clinical studies. Immunogenicity, off-targeting, mutations, delivery techniques, and ethics are main difficulties with the CRISPR/Cas9 technology (Fig. 3).

Immunogenicity

Immunogenicity or immunoreactivity refers to biological reactions in which the biomaterials being recognized by the body's immune system as a foreign agent and provoke immunological responses resulting in severe adverse reactions [162]. Researchers have found that pre-exposure to similar antigens of the CRISPR systems components such as Cas9, delivery vectors (e.g., adenoviral vectors), and guide RNAs can induce immune reactions, which may lead to failure in successful genome editing [163–165]. It has been stated that CRISPR systems have ability to trigger both innate immunity (by guide RNAs) and/or acquired immune responses (anti-Cas9 antibody in humoral responses and anti-Cas9 T cells in cellular responses) in healthy individuals. To conquer this challenge, several strategies have been proposed including phosphatase treatment of in vitro–transcribed guide RNAs, developing novel Cas proteins and delivery vectors with lower possibility of prior exposure in individuals, and evaluating and monitoring the immune reactions toward the CRISPR systems components before and during clinical trials [163–166].

Off-Targeting

Off-targeting is a major problem with CRISPR/Cas9. When using the CRISPR–Cas9 system in complex genomic species, such as mammals, the gRNA may bind to non-specific region owing to similarities in the genome, which may lead to subsequent mutations. Bioinformatics technologies have been created to anticipate and decrease off-target alterations. These could be upgraded to help researchers create novel medicines [167], [10, 11, 168]. Utilizing the High-fidelity SpCas9 can also increase efficiency and decrease off-target functions. In HDR-based repair, longer gRNA often provides a stronger affinity for target genes. Moreover, enzymes (nucleases) can be modified, or Cas9 can be expressed temporarily, to reduce the possibility of off-target consequences. Besides, the type of vectors can diminish the off-target risk, for instance, no integration into the host DNA occurs when we employ adeno virus (AV) vectors [8, 169, 170].

Mutations

Contrary to other hereditary disorders like hemophilia or Huntington diseases, cancer is linked to a variety of mutations in different genes [171, 172–174]. To achieve the desired outcome in cancer therapy by knocking in the mutant gene, one strategy that should be considered is modification of the aberrant nucleotides of tumor suppressor genes. By correcting the activity of these tumor suppressor genes, the CRISPR/Cas9 technology has ability to limit or diminish

carcinogenesis [175]. Besides, the CRISPR–Cas system can alter the nucleotide sequences to block the function of oncogenes like KRAS [175–178]. However, in cancer gene therapy, altering a single mutant nucleotide is seldom adequate. Correcting mutant nucleotides by knocking in is more difficult in CRISPR–Cas9 than knocking out and as mentioned above, cancer is aroused by several mutations in multiple genes. Therefore, knocking in all of the cancer-causing genes takes longer, requires multi-guide RNAs, and has greater chance of off-targeting, which make it laborious [179]. To overcome the obstacles, we can conduct CHyM-ErA methods which relies on two Cas proteins, Cas9 and Cas12a nucleases, and use bioinformatic tools to recognize interaction sites of proteins and predict the outcome of CRISPR knock-out [180–182].

Delivery Techniques

The choice of a safe, accurate delivery mechanism to transfer CRISPR into the tumor location, especially in vivo, and targeting the appropriate sequence inside the nucleus are further challenges. CRISPR/Cas9 is supplied by viral, physical, and extracellular vesicle-base systems. Each approach has advantages and limitations. The choice of the proper vector involves packaging, transportation, and site targeting. Viral vectors are employed in vivo and in vitro, but have drawbacks such as immunological response and insertional constraint. After introducing a viral vector in vivo, it is exposed for a long period, increasing the risk of mutations and off-targeting [183–186]. Another possible solution is employing new delivery methods such as solid lipid nanoparticle, gold nanoclusters and nanowires, and cationic lipids [187–191]. However, each one has its own concerns, such as difficulties in producing large amounts of nanoparticle for clinical applications and toxicity of cationic lipids [185, 192].

Ethics

CRISPR/Cas9-mediated genome editing has shed fresh light on human genetic changes. Given the unpredictability and far-reaching impacts of this technology applications, its ethical and societal ramifications must be thoroughly examined [193].

Conclusion

The substantial achievements in gene-editing technology in recent years are already making dramatic advances in improving human health, such as using gene editors in ongoing clinical trials to treat various types of human disorders, including HIV, cancer, and hematologic disorders. As gene editors progress, new treatments for other disorders will likely come
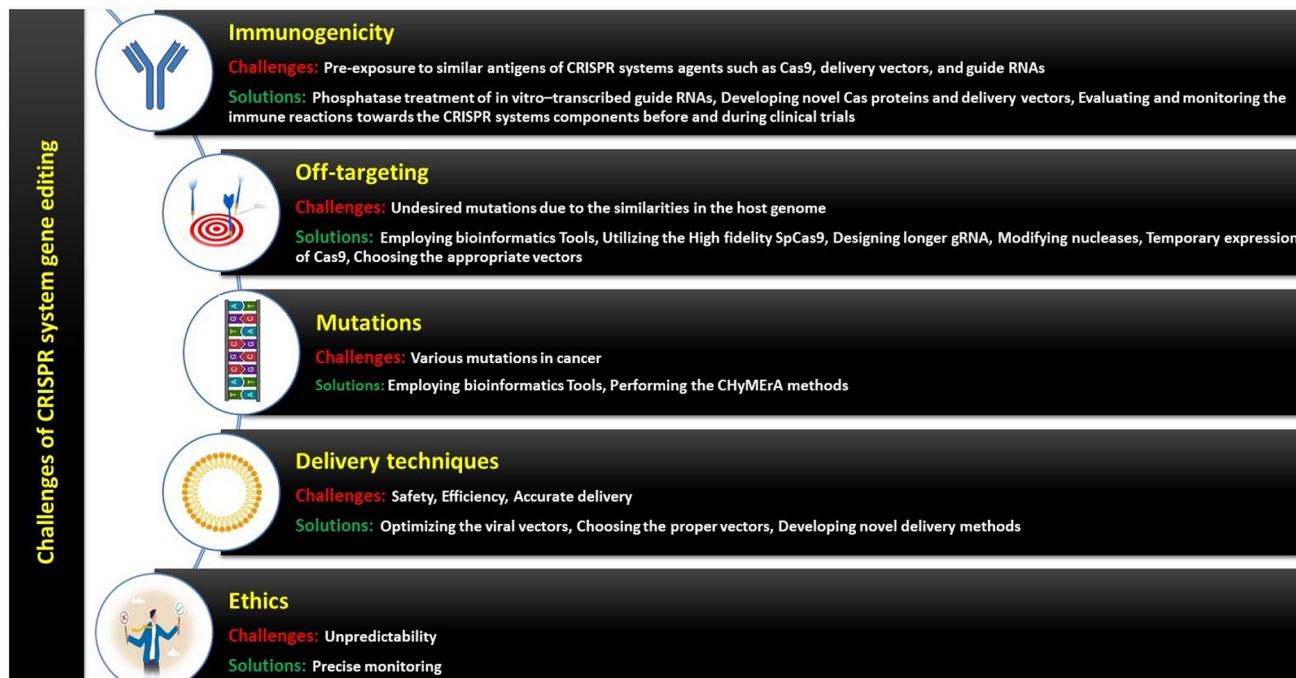
Fig. 3 Major challenges and possible approaches toward utilizing the CRISPR–Cas in clinical settings

into view. Specifically, the CRISPR systems as modern gene editors are rapidly progressing and have been used to induce different genetic modifications in human cells, such as site-specific editing of DNA sequences, activation or suppression of gene expression of interest, and epigenetic changes. Nevertheless, despite the potential concessions of gene editors for human therapy, the basic biology underlying these gene-editing tools should be better understood to ensure the safety and efficiency of treatment options for affected individuals. Many CRISPR systems have been examined only in vitro, and their effectiveness and safety in vivo are still unclear. This will probably involve the complicated interaction between a given tool's molecular function and delivery methods. Some CRISPR ingredients have elicited immunogenic responses in human. In many cases, there are widespread off-target effects of using CRISPR-based gene-editing tools, which can be hazardous if we have to use them in a critical organ or if they are inadvertently delivered. The novel routes for enhancing delivery to desired sites and reduction of off-target sites should be investigated. These are issues that are now being fully explored by research teams, and advances in these areas will be essential to the outcomes of gene-editing therapies. There are also many ethical issues associated with the rapid growth of new gene-editing tools in recent years, from editing the developing human embryo to determining who has the authority to make the regulations or who should make the decisions. There will be a need for global debate between science and politics to control and manage the use of CRISPR and genome editing technologies in developing infants. In addition, health and

disease often include a range, contrary to popular belief and thus, decisions must be made based on a known or potential compromise. Moreover Genome editing has significant risks; therefore, a balance must be struck between the risks accepted and the significant benefits in each case. This balance will be altered as technology advances and the risk/benefit status for a given treatment changes. Lastly, as novel technologies develop, there is the concern that they may be misused by accident or purposely. Gene-editing technologies, especially CRISPR, are potent and have great potential to deliver new therapeutic opportunities for many human disorders. Since the extent of funds allocated to better understanding and describing these technologies increases each year, their clinical expansion is very close to becoming a reality. Finally, CRISPR/Cas9 cell-based regeneration therapies offer the potential to overcome the rejection problems associated with transplantation procedures, which requires donor compatibility. These procedures, known as autologous therapies, entail genetic modification to repair a mutation in a patient's own tissues.

Declarations

Competing interests The authors declare no competing interests.

References

1. Guha, T. K., Wai, A., & Hausner, G. (2017). Programmable genome editing tools and their regulation for efficient genome engineering. *Computational and structural biotechnology journal, 15*, 146–160.

2. Tafazoli, A., Behjati, F., Farhud, D. D., & Abbaszadegan, M. R. (2019). Combination of genetics and nanotechnology for down syndrome modification: A potential hypothesis and review of the literature. *Iranian Journal of Public Health, 48*(3), 371.

3. Lippow, S. M., Aha, P. M., Parker, M. H., Blake, W. J., Baynes, B. M., & Lipovšek, D. (2009). Creation of a type IIS restriction endonuclease with a long recognition sequence. *Nucleic acids research, 37*(9), 3061–3073.

4. Adli, M. (2018). The CRISPR tool kit for genome editing and beyond. *Nature communications, 9*(1), 1–13.

5. Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A., & Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science, 315*(5819), 1709–1712.

6. Mojica, F. J., Díez-Villaseñor, C., Soria, E., & Juez, G. (2000). Biological significance of a family of regularly spaced repeats in the genomes of Archaea. *Bacteria and mitochondria. Molecular microbiology, 36*(1), 244–246.

7. Sternberg, S. H., & Doudna, J. A. (2015). Expanding the biologist's toolkit with CRISPR-Cas9. *Molecular cell, 58*(4), 568–574.

8. Bhattacharjee, G., Gohil, N., Khambhati, K., Mani, I., Maurya, R., Karapurkar, J. K., Gohil, J., Chu, D.-T., Vu-Thi, H., Alzahrani, K. J., Show, P.-L., Rawal, R. M., Ramakrishna, S., & Singh, V. (2022). Current approaches in CRISPR-Cas9 mediated gene editing for biomedical and therapeutic applications. *Journal of Controlled Release, 343*, 703–723. https://doi.org/10.1016/j.jconrel.2022.02.005

9. Aman, R., Mahas, A., & Mahfouz, M. (2020). Nucleic acid detection using CRISPR/cas biosensing technologies. *ACS Synthetic Biology, 9*(6), 1226–1233. https://doi.org/10.1021/acssynbio.9b00507

10. Li, D., Zhou, H., & Zeng, X. (2019). Battling CRISPR-Cas9 off-target genome editing. *Cell Biology and Toxicology, 35*, 403–405.

11. Li, Y., Li, S., Wang, J., & Liu, G. (2019). CRISPR/Cas systems towards next-generation biosensing. *Trends in Biotechnology, 37*(7), 730–743. https://doi.org/10.1016/j.tibtech.2018.12.005

12. Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., & Marraffini, L. A. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science, 339*(6121), 819–823.

13. Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., Norville, J. E., & Church, G. M. (2013). RNA-guided human genome engineering via Cas9. *Science, 339*(6121), 823–826.

14. Wiedenheft, B., Sternberg, S. H., & Doudna, J. A. (2012). RNA-guided genetic silencing systems in bacteria and archaea. *Nature, 482*(7385), 331–338.

15. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science, 337*(6096), 816–821.

16. Gaj, T., Gersbach, C. A., & Barbas, C. F., III. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends in biotechnology, 31*(7), 397–405.

17. Lino, C. A., Harper, J. C., Carney, J. P., & Timlin, J. A. (2018). Delivering CRISPR: A review of the challenges and approaches. *Drug Delivery, 25*(1), 1234–1257.

18. Makarova, K. S., Wolf, Y. I., Iranzo, J., Shmakov, S. A., Alkhnbashi, O. S., Brouns, S. J., Charpentier, E., Cheng, D., Haft, D. H., & Horvath, P. (2020). Evolutionary classification of CRISPR–Cas systems: A burst of class 2 and derived variants. *Nature Reviews Microbiology, 18*(2), 67–83.

19. Mohanraju, P., Makarova, K. S., Zetsche, B., Zhang, F., Koonin, E. V., & Van der Oost, J. (2016). Diverse evolutionary roots and mechanistic variations of the CRISPR-Cas systems. *Science*. https://doi.org/10.1126/science.aad5147

20. Charpentier, E., Richter, H., van der Oost, J., & White, M. F. (2015). Biogenesis pathways of RNA guides in archaeal and bacterial CRISPR-Cas adaptive immunity. *FEMS microbiology reviews, 39*(3), 428–441.

21. Koonin, E. V., Makarova, K. S., & Zhang, F. (2017). Diversity, classification and evolution of CRISPR-Cas systems. *Current opinion in microbiology, 37*, 67–78.

22. Makarova, K. S., Wolf, Y. I., Alkhnbashi, O. S., Costa, F., Shah, S. A., Saunders, S. J., Barrangou, R., Brouns, S. J., Charpentier, E., & Haft, D. H. (2015). An updated evolutionary classification of CRISPR–Cas systems. *Nature Reviews Microbiology, 13*(11), 722–736.

23. Moon, S. B., Kim, D. Y., Ko, J.-H., & Kim, Y.-S. (2019). Recent advances in the CRISPR genome editing tool set. *Experimental & molecular medicine, 51*(11), 1–11.

24. Nidhi, S., Anand, U., Oleksak, P., Tripathi, P., Lal, J. A., Thomas, G., Kuca, K., & Tripathi, V. (2021). Novel CRISPR–Cas systems: An updated review of the current achievements, applications, and future research perspectives. *International journal of molecular sciences, 22*(7), 3327.

25. Özcan, A., Pausch, P., Linden, A., Wulf, A., Schühle, K., Heider, J., Urlaub, H., Heimerl, T., Bange, G., & Randau, L. (2019). Type IV CRISPR RNA processing and effector complex formation in Aromatoleum aromaticum. *Nature microbiology, 4*(1), 89–96.

26. Cebrian-Serrano, A., & Davies, B. (2017). CRISPR-Cas orthologues and variants: Optimizing the repertoire, specificity and delivery of genome engineering tools. *Mammalian Genome, 28*(7), 247–261.

27. Chylinski, K., Makarova, K. S., Charpentier, E., & Koonin, E. V. (2014). Classification and evolution of type II CRISPR-Cas systems. *Nucleic acids research, 42*(10), 6091–6105.

28. Mir, A., Edraki, A., Lee, J., & Sontheimer, E. J. (2018). Type II-C CRISPR-Cas9 biology, mechanism, and application. *ACS chemical biology, 13*(2), 357–365.

29. Mougiakos, I., Bosma, E. F., de Vos, W. M., van Kranenburg, R., & van der Oost, J. (2016). Next generation prokaryotic engineering: The CRISPR-Cas toolkit. *Trends in biotechnology, 34*(7), 575–587.

30. Sternberg, S. H., LaFrance, B., Kaplan, M., & Doudna, J. A. (2015). Conformational control of DNA target cleavage by CRISPR–Cas9. *Nature, 527*(7576), 110–113.

31. Szczelkun, M. D., Tikhomirova, M. S., Sinkunas, T., Gasiunas, G., Karvelis, T., Pschera, P., Siksnys, V., & Seidel, R. (2014). Direct observation of R-loop formation by single RNA-guided Cas9 and Cascade effector complexes. *Proceedings of the National Academy of Sciences, 111*(27), 9798–9803.

32. Truong, D.-J.J., Kühner, K., Kühn, R., Werfel, S., Engelhardt, S., Wurst, W., & Ortiz, O. (2015). Development of an intein-mediated split–Cas9 system for gene therapy. *Nucleic acids research, 43*(13), 6450–6458.

33. Zuo, Z., & Liu, J. (2016). Cas9-catalyzed DNA cleavage generates staggered ends: Evidence from molecular dynamics simulations. *Scientific reports, 6*(1), 1–9.

34. Fernandes, H., Pastor, M., & Bochtler, M. (2016). Type II and type V CRISPR effector nucleases from a structural biologistâ s perspective. *Postępy Biochemii, 62*(3), 315–326.

35. Fonfara, I., Richter, H., Bratovič, M., Le Rhun, A., & Charpentier, E. (2016). The CRISPR-associated DNA-cleaving enzyme

Cpf1 also processes precursor CRISPR RNA. *Nature, 532*(7600), 517–521.

36. Harrington, L. B., Burstein, D., Chen, J. S., Paez-Espino, D., Ma, E., Witte, I. P., Cofsky, J. C., Kyrpides, N. C., Banfield, J. F., & Doudna, J. A. (2018). Programmed DNA destruction by miniature CRISPR-Cas14 enzymes. *Science, 362*(6416), 839–842.

37. Karvelis, T., Bigelyte, G., Young, J. K., Hou, Z., Zedaveinyte, R., Budre, K., Paulraj, S., Djukanovic, V., Gasior, S., & Silanskas, A. (2020). PAM recognition by miniature CRISPR–Cas12f nucleases triggers programmable double-stranded DNA target cleavage. *Nucleic acids research, 48*(9), 5016–5023.

38. Liu, J.-J., Orlova, N., Oakes, B. L., Ma, E., Spinner, H. B., Baney, K. L., Chuck, J., Tan, D., Knott, G. J., & Harrington, L. B. (2019). CasX enzymes comprise a distinct family of RNA-guided genome editors. *Nature, 566*(7743), 218–223.

39. Liu, L., Chen, P., Wang, M., Li, X., Wang, J., Yin, M., & Wang, Y. (2017). C2c1-sgRNA complex structure reveals RNA-guided DNA cleavage mechanism. *Molecular cell, 65*(2), 310–322.

40. Strecker, J., Jones, S., Koopal, B., Schmid-Burgk, J., Zetsche, B., Gao, L., Makarova, K. S., Koonin, E. V., & Zhang, F. (2019). Engineering of CRISPR-Cas12b for human genome editing. *Nature communications, 10*(1), 1–8.

41. Swarts, D. C., van der Oost, J., & Jinek, M. (2017). Structural basis for guide RNA processing and seed-dependent DNA targeting by CRISPR-Cas12a. *Molecular cell, 66*(2), 221–233.

42. Teng, F., Cui, T., Feng, G., Guo, L., Xu, K., Gao, Q., Li, T., Li, J., Zhou, Q., & Li, W. (2018). Repurposing CRISPR-Cas12b for mammalian genome engineering. *Cell discovery, 4*(1), 1–15.

43. Yamano, T., Nishimasu, H., Zetsche, B., Hirano, H., Slaymaker, I. M., Li, Y., Fedorova, I., Nakane, T., Makarova, K. S., & Koonin, E. V. (2016). Crystal structure of Cpf1 in complex with guide RNA and target DNA. *Cell, 165*(4), 949–962.

44. Yan, W. X., Hunnewell, P., Alfonse, L. E., Carte, J. M., Keston-Smith, E., Sothiselvam, S., Garrity, A. J., Chong, S., Makarova, K. S., & Koonin, E. V. (2019). Functionally diverse type V CRISPR-Cas systems. *Science, 363*(6422), 88–91.

45. Yang, H., Gao, P., Rajashankar, K. R., & Patel, D. J. (2016). PAM-dependent target DNA recognition and cleavage by C2c1 CRISPR-Cas endonuclease. *Cell, 167*(7), 1814–1828.

46. Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., Slaymaker, I. M., Makarova, K. S., Essletzbichler, P., Volz, S. E., Joung, J., Van Der Oost, J., & Regev, A. (2015). Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell, 163*(3), 759–771.

47. Abudayyeh, O. O., Gootenberg, J. S., Konermann, S., Joung, J., Slaymaker, I. M., Cox, D. B., Shmakov, S., Makarova, K. S., Semenova, E., & Minakhin, L. (2016). C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science*. https://doi.org/10.1126/science.aaf5573

48. East-Seletsky, A., O'Connell, M. R., Burstein, D., Knott, G. J., & Doudna, J. A. (2017). RNA targeting by functionally orthogonal type VI-A CRISPR-Cas enzymes. *Molecular cell, 66*(3), 373–383.

49. Gootenberg, J. S., Abudayyeh, O. O., Lee, J. W., Essletzbichler, P., Dy, A. J., Joung, J., Verdine, V., Donghia, N., Daringer, N. M., & Freije, C. A. (2017). Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science, 356*(6336), 438–442.

50. O'Connell, M. R. (2019). Molecular mechanisms of RNA targeting by Cas13-containing type VI CRISPR–Cas systems. *Journal of molecular biology, 431*(1), 66–87.

51. Huang, C.-H., Lee, K.-C., & Doudna, J. A. (2018). Applications of CRISPR-Cas enzymes in cancer therapeutics and detection. *Trends in cancer, 4*(7), 499–512.

52. Jiang, C., Meng, L., Yang, B., & Luo, X. (2020). Application of CRISPR/Cas9 gene editing technique in the study of cancer treatment. *Clinical genetics, 97*(1), 73–88.

53. Ou, X., Ma, Q., Yin, W., Ma, X., & He, Z. (2021). CRISPR/Cas9 gene-editing in cancer immunotherapy: Promoting the present revolution in cancer therapy and exploring more. *Frontiers in Cell and Developmental Biology*. https://doi.org/10.3389/fcell.2021.674467

54. Baylis, F., & McLeod, M. (2017). First-in-human phase 1 CRISPR gene editing cancer trials: Are we ready? *Current gene therapy, 17*(4), 309–319.

55. He, S. (2020). The first human trial of CRISPR-based cell therapy clears safety concerns as new treatment for late-stage lung cancer. *Signal Transduction and Targeted Therapy, 5*(1), 1–2.

56. Ma, P., Meng, Q., Sun, B., Zhao, B., Dang, L., Zhong, M., Liu, S., Xu, H., Mei, H., & Liu, J. (2020). MeCas12a, a highly sensitive and specific system for COVID-19 detection. *Advanced science, 7*(20), 2001300.

57. Lin, H., Li, G., Peng, X., Deng, A., Ye, L., Shi, L., Wang, T., & He, J. (2021). the use of CRISPR/cas9 as a tool to study human infectious viruses. *Frontiers in Cellular and Infection Microbiology*. https://doi.org/10.3389/fcimb.2021.590989

58. Singh, V., Gohil, N., Ramirez Garcia, R., Braddick, D., & Fofié, C. K. (2018). Recent advances in CRISPR-Cas9 genome editing technology for biological and biomedical investigations. *Journal of cellular biochemistry, 119*(1), 81–94.

59. Bui, L. M., Phung, H. T. T., Thi, T. T. H., Singh, V., Maurya, R., Khambhati, K., Chia-Ching, W., Uddin, M. J., Trung, D. M., & Chu, D. T. (2021). Recent findings and applications of biomedical engineering for COVID-19 diagnosis: A critical review. *Bioengineered, 12*(1), 8594–8613.

60. Bebenek, I., Bannister, R., Dubinion, J., Fortin, M., Liu, M., Motter, A. L., Rohde, C. M., & Wrzesinski, C. (2022). COVID-19 Therapeutics and Vaccines: A Race to Save Lives. *Toxicological Sciences, 185*(2), 119–127.

61. Mayuramart, O., Nimsamer, P., Rattanaburi, S., Chantaravisoot, N., Khongnomnan, K., Chansaenroj, J., Puenpa, J., Suntronwong, N., Vichaiwattana, P., & Poovorawan, Y. (2021). Detection of severe acute respiratory syndrome coronavirus 2 and influenza viruses based on CRISPR-Cas12a. *Experimental Biology and Medicine, 246*(4), 400–405.

62. Ebrahimi, S., Khanbabaei, H., Abbasi, S., Fani, M., Soltani, S., Zandi, M., & Najafimemar, Z. (2022). CRISPR-cas system: A promising diagnostic tool for covid-19. *Avicenna Journal of Medical Biotechnology, 14*(1), 3.

63. Schultze, J. L., & Aschenbrenner, A. C. (2021). COVID-19 and the human innate immune system. *Cell, 184*(7), 1671–1692.

64. Zhang, B., Yue, D., Wang, Y., Wang, F., Wu, S., & Hou, H. (2021). The dynamics of immune response in COVID-19 patients with different illness severity. *Journal of Medical Virology, 93*(2), 1070–1077.

65. He, X., & Zeng, X. X. (2022). Immunotherapy and CRISPR Cas Systems: Potential Cure of COVID-19? Drug Design. *Development and Therapy, 16*, 951.

66. Luthra, R., Kaur, S., & Bhandari, K. (2021). Applications of CRISPR as a potential therapeutic. *Life Sciences, 284*, 119908.

67. Zeng, L., Liu, Y., Nguyenla, X. H., Abbott, T. R., Han, M., Zhu, Y., Chemparathy, A., Lin, X., Chen, X., & Wang, H. (2022). Broad-spectrum CRISPR-mediated inhibition of SARS-CoV-2 variants and endemic coronaviruses in vitro. *Nature Communications, 13*(1), 1–16.

68. Dampier, W., Sullivan, N. T., Mell, J. C., Pirrone, V., Ehrlich, G. D., Chung, C.-H., Allen, A. G., DeSimone, M., Zhong, W., & Kercher, K. (2018). Broad-spectrum and personalized guide RNAs for CRISPR/Cas9 HIV-1 therapeutics. *AIDS research and human retroviruses, 34*(11), 950–960.

69. Liu, Y., Jeeninga, R. E., Klaver, B., Berkhout, B., & Das, A. T. (2021). Transient CRISPR-Cas treatment can prevent reactivation

of hiv-1 replication in a latently infected T-cell line. *Viruses, 13*(12), 2461.

70. Xu, L., Yang, H., Gao, Y., Chen, Z., Xie, L., Liu, Y., Liu, Y., Wang, X., Li, H., & Lai, W. (2017). CRISPR/Cas9-mediated CCR5 ablation in human hematopoietic stem/progenitor cells confers HIV-1 resistance in vivo. *Molecular Therapy, 25*(8), 1782–1789.

71. Hou, P., Chen, S., Wang, S., Yu, X., Chen, Y., Jiang, M., Zhuang, K., Ho, W., Hou, W., Huang, J., & Guo, D. (2015). Genome editing of CXCR4 by CRISPR/cas9 confers cells resistant to HIV-1 infection. *Science and Reports, 5*, 15577. https://doi.org/10.1038/srep15577

72. Cornu, T. I., Mussolino, C., Müller, M. C., Wehr, C., Kern, W. V., & Cathomen, T. (2021). HIV Gene therapy: An update. *Human Gene Therapy, 32*(1–2), 52–65.

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

74. David, C. (2016). CRISPR gene editing tested in a person. *Nature, 539*, 479.

75. Liu, S. (2020). Legal reflections on the case of genomeedited babies. *Global Health Research and Policy*. https://doi.org/10.1186/s41256-020-00153-4

76. Gao, C., Wu, P., Yu, L., Liu, L., Liu, H., Tan, X., Wang, L., Huang, X., & Wang, H. (2022). The application of CRISPR/Cas9 system in cervical carcinogenesis. *Cancer Gene Therapy, 29*(5), 466–474.

77. Hu, Z., Yu, L., Zhu, D., Ding, W., Wang, X., Zhang, C., Wang, L., Jiang, X., Shen, H., & He, D. (2014). Disruption of HPV16-E7 by CRISPR/Cas system induces apoptosis and growth inhibition in HPV16 positive human cervical cancer cells. *BioMedicine Research International*. https://doi.org/10.1155/2014/612823

78. Kennedy, E. M., Kornepati, A. V., Goldstein, M., Bogerd, H. P., Poling, B. C., Whisnant, A. W., Kastan, M. B., & Cullen, B. R. (2014). Inactivation of the human papillomavirus E6 or E7 gene in cervical carcinoma cells by using a bacterial CRISPR/Cas RNA-guided endonuclease. *Journal of virology, 88*(20), 11965–11972.

79. Gao, C., Wu, P., Yu, L., Liu, L., Liu, H., Tan, X., Wang, L., Huang, X., & Wang, H. (2021). The application of CRISPR/Cas9 system in cervical carcinogenesis. *Cancer Gene Therapy*. https://doi.org/10.1038/s41417-021-00366-w

80. Yu, L., Wang, X., Zhu, D., Ding, W., Wang, L., Zhang, C., Jiang, X., Shen, H., Liao, S., & Ma, D. (2014). Disruption of human papillomavirus 16 E6 gene by clustered regularly interspaced short palindromic repeat/Cas system in human cervical cancer cells. *OncoTargets and therapy*. https://doi.org/10.2147/OTT.S64092

81. Ehrke-Schulz, E., Heinemann, S., Schulte, L., Schiwon, M., & Ehrhardt, A. (2020). Adenoviral vectors armed with PAPILLOMAVIRUs oncogene specific CRISPR/Cas9 kill human-papillomavirus-induced cervical cancer cells. *Cancers, 12*(7), 1934.

82. Xiong, J., Tan, S., Yu, L., Shen, H., Qu, S., Zhang, C., Ren, C., Zhu, D., & Wang, H. (2021). E7-Targeted Nanotherapeutics for Key HPV Afflicted Cervical Lesions by Employing CRISPR/Cas9 and Poly (Beta-Amino Ester). *International journal of nanomedicine, 16*, 7609.

83. Zhen, S., Lu, J.-J., Wang, L.-J., Sun, X.-M., Zhang, J.-Q., Li, X., Luo, W.-J., & Zhao, L. (2016). In vitro and in vivo synergistic therapeutic effect of cisplatin with human papillomavirus16 E6/E7 CRISPR/Cas9 on cervical cancer cell line. *Translational oncology, 9*(6), 498–504.

84. Bauer, D. E., & Orkin, S. H. (2015). Hemoglobin switching's surprise: The versatile transcription factor BCL11A is a master repressor of fetal hemoglobin. *Current opinion in genetics & development, 33*, 62–70.

85. Piel, F. B., Steinberg, M. H., & Rees, D. C. (2017). Sickle cell disease. *New England Journal of Medicine, 376*(16), 1561–1573.

86. Saraf, S. L., Molokie, R. E., Nouraie, M., Sable, C. A., Luchtman-Jones, L., Ensing, G. J., Campbell, A. D., Rana, S. R., Niu, X. M., & Machado, R. F. (2014). Differences in the clinical and genotypic presentation of sickle cell disease around the world. *Paediatric respiratory reviews, 15*(1), 4–12.

87. Giardine, B., van Baal, S., Kaimakis, P., Riemer, C., Miller, W., Samara, M., Kollia, P., Anagnou, N. P., Chui, D. H., & Wajcman, H. (2007). HbVar database of human hemoglobin variants and thalassemia mutations: 2007 update. *Human Mutation, 28*(2), 206–206.

88. Thein, S. L. (1998). 3 β-Thalassaemia. *Baillière's clinical haematology, 11*(1), 91–126.

89. Weatherall, D. (1998). 4 Pathophysiology of thalassaemia. *Baillière's clinical haematology, 11*(1), 127–146.

90. Haidar, R., Mhaidli, H., & Taher, A. T. (2010). Paraspinal extramedullary hematopoiesis in patients with thalassemia intermedia. *European Spine Journal, 19*(6), 871–878.

91. Motta, I., Bou-Fakhredin, R., Taher, A. T., & Cappellini, M. D. (2020). Beta thalassemia: New therapeutic options beyond transfusion and iron chelation. *Drugs, 80*(11), 1053–1063.

92. Taher, A. T., Weatherall, D. J., & Cappellini, M. D. (2018). Thalassaemia. *The Lancet, 391*(10116), 155–167.

93. Rattananon, P., Anurathapan, U., Bhukhai, K., & Hongeng, S. (2021). The future of gene therapy for transfusion-dependent beta-thalassemia: The power of the lentiviral vector for genetically modified hematopoietic stem cells. *Frontiers in Pharmacology*. https://doi.org/10.3389/fphar.2021.730873

94. Niu, X., He, W., Song, B., Ou, Z., Fan, D., Chen, Y., Fan, Y., & Sun, X. (2016). Combining single strand oligodeoxynucleotides and CRISPR/Cas9 to correct gene mutations in β-thalassemia-induced pluripotent stem cells. *Journal of Biological Chemistry, 291*(32), 16576–16585.

95. Song, B., Fan, Y., He, W., Zhu, D., Niu, X., Wang, D., Ou, Z., Luo, M., & Sun, X. (2015). Improved hematopoietic differentiation efficiency of gene-corrected beta-thalassemia induced pluripotent stem cells by CRISPR/Cas9 system. *Stem cells and development, 24*(9), 1053–1065.

96. Zhang, H., & McCarty, N. (2016). CRISPR-Cas9 technology and its application in haematological disorders. *British journal of haematology, 175*(2), 208–225.

97. Grevet, J. D., Lan, X., Hamagami, N., Edwards, C. R., Sankaranarayanan, L., Ji, X., Bhardwaj, S. K., Face, C. J., Posocco, D. F., & Abdulmalik, O. (2018). Domain-focused CRISPR screen identifies HRI as a fetal hemoglobin regulator in human erythroid cells. *Science, 361*(6399), 285–290.

98. Sankaran, V. G., Menne, T. F., Xu, J., Akie, T. E., Lettre, G., Van Handel, B., Mikkola, H. K., Hirschhorn, J. N., Cantor, A. B., & Orkin, S. H. (2008). Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor BCL11A. *Science, 322*(5909), 1839–1842.

99. Jensen, T. I., Axelgaard, E., & Bak, R. O. (2019). Therapeutic gene editing in haematological disorders with CRISPR/Cas9. *British journal of haematology, 185*(5), 821–835.

100. Safety, A. (2018). Efficacy Study Evaluating CTX001 in Subjects With Severe Sickle Cell Disease. *Vertex* Pharmaceuticals Incorporated. Available online at: https://clinicaltrials.gov/ct2/show/NCT03745287

101. Frangoul, H., Altshuler, D., Cappellini, M. D., Chen, Y.-S., Domm, J., Eustace, B. K., Foell, J., de la Fuente, J., Grupp, S., & Handgretinger, R. (2021). CRISPR-Cas9 gene editing for sickle cell disease and β-thalassemia. *New England Journal of Medicine, 384*(3), 252–260.

102. Musallam, K. M., Sankaran, V. G., Cappellini, M. D., Duca, L., Nathan, D. G., & Taher, A. T. (2012). Fetal hemoglobin levels and morbidity in untransfused patients with β-thalassemia intermedia. *Blood, The Journal of the American Society of Hematology, 119*(2), 364–367.

103. Platt, O. S., Brambilla, D. J., Rosse, W. F., Milner, P. F., Castro, O., Steinberg, M. H., & Klug, P. P. (1994). Mortality in sickle cell disease–life expectancy and risk factors for early death. *New England Journal of Medicine, 330*(23), 1639–1644.

104. Powars, D. R., Weiss, J. N., Chan, L. S., & Schroeder, W. (1984). Is there a threshold level of fetal hemoglobin that ameliorates morbidity in sickle cell anemia? *Blood.* https://doi.org/10.1182/blood.V63.4.921.bloodjournal634921

105. Meneghini, V., Peviani, M., Luciani, M., Zambonini, G., & Gritti, A. (2021). Delivery Platforms for CRISPR/Cas9 Genome Editing of Glial Cells in the Central Nervous System. *Frontiers in Genome Editing.* https://doi.org/10.3389/fgeed.2021.644319

106. Laing, N. G. (2012). Genetics of neuromuscular disorders. *Critical Reviews in Clinical Laboratory Sciences, 49*(2), 33–48. https://doi.org/10.3109/10408363.2012.658906

107. Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., & Nakata, A. (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *Journal of bacteriology, 169*(12), 5429–5433.

108. Cyranoski, D. (2016). CRISPR gene-editing tested in a person for the first time. *Nature news, 539*(7630), 479.

109. Ginn, S. L., Amaya, A. K., Alexander, I. E., Edelstein, M., & Abedi, M. R. (2018). Gene therapy clinical trials worldwide to 2017: An update. *The journal of gene medicine, 20*(5), e3015.

110. Salmaninejad, A., Jafari Abarghan, Y., Bozorg Qomi, S., Bayat, H., Yousefi, M., Azhdari, S., Talebi, S., & Mojarrad, M. (2021). Common therapeutic advances for Duchenne muscular dystrophy (DMD). *International Journal of Neuroscience, 131*(4), 370–389.

111. Long, C., McAnally, J. R., Shelton, J. M., Mireault, A. A., Bassel-Duby, R., & Olson, E. N. (2014). Prevention of muscular dystrophy in mice by CRISPR/Cas9–mediated editing of germline DNA. *Science, 345*(6201), 1184–1188.

112. Chemello, F., Chai, A., Li, H., Rodriguez-Caycedo, C., Sanchez-Ortiz, E., Atmanli, A., Mireault, A., Liu, N., Bassel-Duby, R., & Olson, E. (2021). Precise correction of Duchenne muscular dystrophy exon deletion mutations by base and prime editing. *Science Advances, 7*(18), eabg4910.

113. Xu, L., Zhang, C., Li, H., Wang, P., Gao, Y., Mokadam, N. A., Ma, J., Arnold, W. D., & Han, R. (2021). Efficient precise in vivo base editing in adult dystrophic mice. *Nature communications, 12*(1), 1–14.

114. Levy, J. M., Yeh, W.-H., Pendse, N., Davis, J. R., Hennessey, E., Butcher, R., Koblan, L. W., Comander, J., Liu, Q., & Liu, D. R. (2020). Cytosine and adenine base editing of the brain, liver, retina, heart and skeletal muscle of mice via adeno-associated viruses. *Nature biomedical engineering, 4*(1), 97–110.

115. Deng, H.-X., Zhai, H., Shi, Y., Liu, G., Lowry, J., Liu, B., Ryan, É. B., Yan, J., Yang, Y., & Zhang, N. (2021). Efficacy and long-term safety of CRISPR/Cas9 genome editing in the SOD1-linked mouse models of ALS. *Communications biology, 4*(1), 1–11.

116. Gaj, T., Ojala, D. S., Ekman, F. K., Byrne, L. C., Limsirichai, P., & Schaffer, D. V. (2017). In vivo genome editing improves motor function and extends survival in a mouse model of ALS. *Science Advances, 3*(12), eaar3952.

117. Lim, C. K., Gapinske, M., Brooks, A. K., Woods, W. S., Powell, J. E., Winter, J., Perez-Pinera, P., & Gaj, T. (2020). Treatment of a mouse model of ALS by in vivo base editing. *Molecular Therapy, 28*(4), 1177–1189.

118. Krishnan, G., Zhang, Y., Gu, Y., Kankel, M. W., Gao, F.-B., & Almeida, S. (2020). CRISPR deletion of the C9ORF72 promoter in ALS/FTD patient motor neurons abolishes production of dipeptide repeat proteins and rescues neurodegeneration. *Acta neuropathologica, 140*(1), 81–84.

119. Wu, L., Wang, Y., Du, L., Ji, G., Zhou, R., Zhao, Z., Chen, J., & Zhu, S. (2021). Targeted editing of intronic-splicing silencer enhancement of SMN2 Exon 7 inclusion by CRISPR/Case 9. *Biocell, 45*(6), 1501.

120. Lu, L., Yu, X., Cai, Y., Sun, M., & Yang, H. (2021). Application of CRISPR/Cas9 in alzheimer's disease. *Frontiers in Neuroscience.* https://doi.org/10.3389/fnins.2021.803894

121. Guyon, A., Rousseau, J., Bégin, F.-G., Bertin, T., Lamothe, G., & Tremblay, J. P. (2021). Base editing strategy for insertion of the A673T mutation in the APP gene to prevent the development of AD in vitro. *Molecular Therapy-Nucleic Acids, 24*, 253–263.

122. Patwardhan, A. G., & Belemkar, S. (2021). An update on Alzheimer's disease: Immunotherapeutic agents, stem cell therapy and gene editing. *Life Sciences, 282*, 119790.

123. Raikwar, S. P., Thangavel, R., Dubova, I., Selvakumar, G. P., Ahmed, M. E., Kempuraj, D., Zaheer, S. A., Iyer, S. S., & Zaheer, A. (2019). Targeted gene editing of glia maturation factor in microglia: A novel Alzheimer's disease therapeutic target. *Molecular neurobiology, 56*(1), 378–393.

124. Safieh, M., Korczyn, A. D., & Michaelson, D. M. (2019). ApoE4: An emerging therapeutic target for Alzheimer's disease. *BMC medicine, 17*(1), 1–17.

125. Dabrowska, M., Ciolak, A., Kozlowska, E., Fiszer, A., & Olejniczak, M. (2020). Generation of new isogenic models of Huntington's disease using CRISPR-Cas9 technology. *International journal of molecular sciences, 21*(5), 1854.

126. Dunbar, G. L., Koneru, S., Kolli, N., Sandstrom, M., Maiti, P., & Rossignol, J. (2019). Silencing of the mutant huntingtin gene through CRISPR-Cas9 improves the mitochondrial biomarkers in an in vitro model of Huntington's disease. *Cell Transplantation, 28*(4), 460–463.

127. Monteys, A. M., Ebanks, S. A., Keiser, M. S., & Davidson, B. L. (2017). CRISPR/Cas9 editing of the mutant huntingtin allele in vitro and in vivo. *Molecular Therapy, 25*(1), 12–23.

128. Shin, J. W., Kim, K.-H., Chao, M. J., Atwal, R. S., Gillis, T., MacDonald, M. E., Gusella, J. F., & Lee, J.-M. (2016). Permanent inactivation of Huntington's disease mutation by personalized allele-specific CRISPR/Cas9. *Human molecular genetics, 25*(20), 4566–4576.

129. Yang, S., Chang, R., Yang, H., Zhao, T., Hong, Y., Kong, H. E., Sun, X., Qin, Z., Jin, P., & Li, S. (2017). CRISPR/Cas9-mediated gene editing ameliorates neurotoxicity in mouse model of Huntington's disease. *The Journal of clinical investigation, 127*(7), 2719–2724.

130. Morelli, K. H., Wu, Q., Gosztyla, M. L., Liu, H., Zhang, C., Chen, J., Marina, R., Lee, K., Jones, K. L., & Duan, W. (2022). RNA-Targeting CRISPR/Cas13d system eliminates disease-related phenotypes in pre-clinical models of huntington's disease. *Biorxiv.* https://doi.org/10.1101/2022.01.23.477417

131. Qing, X., Walter, J., Jarazo, J., Arias-Fuenzalida, J., Hillje, A.-L., & Schwamborn, J. C. (2017). CRISPR/Cas9 and piggyBac-mediated footprint-free LRRK2-G2019S knock-in reveals neuronal complexity phenotypes and α-Synuclein modulation in dopaminergic neurons. *Stem Cell Research, 24*, 44–50.

132. Safari, F., Hatam, G., Behbahani, A. B., Rezaei, V., Barekati-Mowahed, M., Petramfar, P., & Khademi, F. (2020). CRISPR system: A high-throughput toolbox for research and treatment of Parkinson's disease. *Cellular and molecular neurobiology, 40*(4), 477–493.

133. Abudayyeh, O. O., Gootenberg, J. S., Essletzbichler, P., Han, S., Joung, J., Belanto, J. J., Verdine, V., Cox, D. B. T., Kellner, M. J.,

Regev, A., Lander, E. S., Voytas, D. F., Ting, A. Y., & Zhang, F. (2017). RNA targeting with CRISPR-Cas13. *Nature, 550*(7675), 280–284. https://doi.org/10.1038/nature24049

134. Gillmore, J. D., Gane, E., Taubel, J., Kao, J., Fontana, M., Maitland, M. L., Seitzer, J., O'Connell, D., Walsh, K. R., Wood, K., Phillips, J., Xu, Y., Amaral, A., Boyd, A. P., Cehelsky, J. E., McKee, M. D., Schiermeier, A., Harari, O., Murphy, A., & Lebwohl, D. (2021). CRISPR-Cas9 in vivo gene editing for transthyretin amyloidosis. *New England Journal Medicine, 385*(6), 493–502. https://doi.org/10.1056/NEJMoa2107454

135. Cho, Y. D., Kim, K. H., Lee, Y. M., Ku, Y., & Seol, Y. J. (2021). Periodontal wound healing and tissue regeneration: A narrative review. *Pharmaceuticals.* https://doi.org/10.3390/ph14050456

136. Selle, K., Fletcher, J. R., Tuson, H., Schmitt, D. S., McMillan, L., Vridhambal, G. S., Rivera, A. J., Montgomery, S. A., Fortier, L. C., Barrangou, R., Theriot, C. M., & Ousterout, D. G. (2020). In Vivo Targeting of Clostridioides difficile Using Phage-Delivered CRISPR-Cas3 Antimicrobials. *MBio.* https://doi.org/10.1128/mBio.00019-20

137. Barbour, A., Glogauer, J., Grinfeld, L., Ostadsharif Memar, R., Fine, N., Tenenbaum, H., & Glogauer, M. (2021). The role of CRISPR-Cas in advancing precision periodontics. *Journal of Periodontal Research, 56*(3), 454–461. https://doi.org/10.1111/jre.12846

138. Yoon, Y., Kim, T. J., Lee, J. M., & Kim, D. Y. (2018). SOD2 is upregulated in periodontitis to reduce further inflammation progression. *Oral Diseases, 24*(8), 1572–1580. https://doi.org/10.1111/odi.12933

139. Zhang, P., Zhang, W., Zhang, D., Wang, M., Aprecio, R., Ji, N., Mohamed, O., Li, Y., Ding, Y., & Wang, Q. (2018). 25-Hydroxyvitamin D(3) -enhanced PTPN2 positively regulates periodontal inflammation through the JAK/STAT pathway in human oral keratinocytes and a mouse model of type 2 diabetes mellitus. *Journal of Periodontal Research, 53*(3), 467–477. https://doi.org/10.1111/jre.12535

140. Adams, D., Ando, Y., Beirão, J. M., Coelho, T., Gertz, M. A., Gillmore, J. D., Hawkins, P. N., Lousada, I., Suhr, O. B., & Merlini, G. (2021). Expert consensus recommendations to improve diagnosis of ATTR amyloidosis with polyneuropathy. *Journal of Neurology, 268*(6), 2109–2122. https://doi.org/10.1007/s00415-019-09688-0

141. Mircsof, D. (2020). Diagnosis of Amyloidosis: A Survey of Current Awareness and Clinical Challenges Among Cardiologists in Switzerland. *Cardiology and therapy, 9*(1), 127–138. https://doi.org/10.1007/s40119-019-00160-8

142. Schönland, S. O., Dreger, P., de Witte, T., & Hegenbart, U. (2012). Current status of hematopoietic cell transplantation in the treatment of systemic amyloid light-chain amyloidosis. *Bone Marrow Transplantation, 47*(7), 895–905. https://doi.org/10.1038/bmt.2011.152

143. Cavaliere, G. (2018). Genome editing and assisted reproduction: Curing embryos, society or prospective parents? *Medicine, Health Care and Philosophy, 21*(2), 215–225. https://doi.org/10.1007/s11019-017-9793-y

144. Ranisch, R. (2020). Germline genome editing versus preimplantation genetic diagnosis: Is there a case in favour of germline interventions? *Bioethics, 34*(1), 60–69. https://doi.org/10.1111/bioe.12635

145. Steffann, J., Jouannet, P., Bonnefont, J. P., Chneiweiss, H., & Frydman, N. (2018). Could failure in preimplantation genetic diagnosis justify editing the human embryo genome? *Cell Stem Cell, 22*(4), 481–482. https://doi.org/10.1016/j.stem.2018.01.004

146. Hammerstein, A. L. V., Eggel, M., & Biller-Andorno, N. (2019). Is selecting better than modifying? An investigation of arguments against germline gene editing as compared to preimplantation

genetic diagnosis. *BMC Medical Ethics, 20*(1), 83. https://doi.org/10.1186/s12910-019-0411-9

147. Foran, M. (2021). Crafting the Perfect Cure? Embryonic CRISPr Editing and Equality of Access . Law School Student Scholarship. 1139.

148. Tang, J. X., Pyle, A., Taylor, R. W., & Oláhová, M. (2021). Interrogating Mitochondrial Biology and Disease Using CRISPR/Cas9 Gene Editing. *Genes, 12*(10), 1604.

149. de Oliveira, V. C., Santos Roballo, K. C., Mariano Junior, C. G., Santos, S. I. P., Bressan, F. F., Chiaratti, M. R., Tucker, E. J., Davis, E. E., Concordet, J.-P., & Ambrósio, C. E. (2022). HEK293T Cells with TFAM Disruption by CRISPR-Cas9 as a model for mitochondrial regulation. *Life, 12*(1), 22.

150. Shinoda, S., Kitagawa, S., Nakagawa, S., Wei, F.-Y., Tomizawa, K., Araki, K., Araki, M., Suzuki, T., & Suzuki, T. (2019). Mammalian NSUN2 introduces 5-methylcytidines into mitochondrial tRNAs. *Nucleic acids research, 47*(16), 8734–8745.

151. Van Haute, L., Lee, S.-Y., McCann, B. J., Powell, C. A., Bansal, D., Vasiliauskaitė, L., Garone, C., Shin, S., Kim, J.-S., & Frye, M. (2019). NSUN2 introduces 5-methylcytosines in mammalian mitochondrial tRNAs. *Nucleic acids research, 47*(16), 8720–8733.

152. Jin, X., Zhang, Z., Nie, Z., Wang, C., Meng, F., Yi, Q., Chen, M., Sun, J., Zou, J., & Jiang, P. (2021). An animal model for mitochondrial tyrosyl-tRNA synthetase deficiency reveals links between oxidative phosphorylation and retinal function. *Journal of Biological Chemistry.* https://doi.org/10.1016/j.jbc.2021.100437

153. Wang, T., Birsoy, K., Hughes, N. W., Krupczak, K. M., Post, Y., Wei, J. J., Lander, E. S., & Sabatini, D. M. (2015). Identification and characterization of essential genes in the human genome. *Science, 350*(6264), 1096–1101.

154. Mendelsohn, B. A., Bennett, N. K., Darch, M. A., Yu, K., Nguyen, M. K., Pucciarelli, D., Nelson, M., Horlbeck, M. A., Gilbert, L. A., & Hyun, W. (2018). A high-throughput screen of real-time ATP levels in individual cells reveals mechanisms of energy failure. *PLoS biology, 16*(8), e2004624.

155. Tian, M., Xia, P., Gou, X., Yan, L., Yu, H., & Zhang, X. (2022). CRISPR screen identified that UGT1A9 was required for bisphenols-induced mitochondria dyshomeostasis. *Environmental Research, 205*, 112427.

156. Mennuni, M., Filograna, R., Felser, A., Bonekamp, N. A., Giavalisco, P., Lytovchenko, O., & Larsson, N. G. (2022). Metabolic resistance to the inhibition of mitochondrial transcription revealed by CRISPR-Cas9 screen. *EMBO reports, 23*(1), e53054.

157. Hoshino, A., Wang, W.-J., Wada, S., McDermott-Roe, C., Evans, C. S., Gosis, B., Morley, M. P., Rathi, K. S., Li, J., & Li, K. (2019). The ADP/ATP translocase drives mitophagy independent of nucleotide exchange. *Nature, 575*(7782), 375–379.

158. Guo, X., Aviles, G., Liu, Y., Tian, R., Unger, B. A., Lin, Y.-H.T., Wiita, A. P., Xu, K., Correia, M. A., & Kampmann, M. (2020). Mitochondrial stress is relayed to the cytosol by an OMA1–DELE1–HRI pathway. *Nature, 579*(7799), 427–432.

159. Jo, A., Ham, S., Lee, G. H., Lee, Y.-I., Kim, S., Lee, Y.-S., Shin, J.-H., & Lee, Y. (2015). Efficient mitochondrial genome editing by CRISPR/Cas9. *BioMedicine Research International.* https://doi.org/10.1155/2015/305716

160. Gómez-Tatay, L., Hernández-Andreu, J. M., & Aznar, J. (2017). Mitochondrial modification techniques and ethical issues. *Journal of clinical medicine, 6*(3), 25.

161. Hussain, S.-R.A., Yalvac, M. E., Khoo, B., Eckardt, S., & McLaughlin, K. J. (2021). Adapting CRISPR/Cas9 system for targeting mitochondrial genome. *Frontiers in Genetics.* https://doi.org/10.3389/fgene.2021.627050

162. Piché, M.-S., Mounho-Zamora, B., LeSauteur, L., & Burns-Naas, L. A. (2015). Chapter 27 - Immunotoxicology Testing of

Monoclonal Antibodies in Macaca fascicularis: From Manufacturing to Preclinical Studies. In J. Bluemel, S. Korte, E. Schenck, & G. F. Weinbauer (Eds.), *The Nonhuman Primate in Nonclinical Drug Development and Safety Assessment.* Cambridgeb: Academic Press. https://doi.org/10.1016/B978-0-12-417144-2.00027-5

163. Charlesworth, C. T., Deshpande, P. S., Dever, D. P., Camarena, J., Lemgart, V. T., Cromer, M. K., Vakulskas, C. A., Collingwood, M. A., Zhang, L., & Bode, N. M. (2019). Identification of preexisting adaptive immunity to Cas9 proteins in humans. *Nature medicine, 25*(2), 249–254.

164. Kim, S., Koo, T., Jee, H.-G., Cho, H.-Y., Lee, G., Lim, D.-G., Shin, H. S., & Kim, J.-S. (2018). CRISPR RNAs trigger innate immune responses in human cells. *Genome Research, 28*(3), 367–373.

165. McCullough, K. T., Boye, S. L., Fajardo, D., Calabro, K., Peterson, J. J., Strang, C. E., Chakraborty, D., Gloskowski, S., Haskett, S., & Samuelsson, S. (2019). Somatic gene editing of GUCY2D by AAV-CRISPR/Cas9 alters retinal structure and function in mouse and macaque. *Human gene therapy, 30*(5), 571–589.

166. Chew, W. L. (2018). Immunity to CRISPR Cas9 and Cas12a therapeutics. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine, 10*(1), e1408.

167. Kang, S.-H., Lee, W.-J., An, J.-H., Lee, J.-H., Kim, Y.-H., Kim, H., Oh, Y., Park, Y.-H., Jin, Y. B., & Jun, B.-H. (2020). Prediction-based highly sensitive CRISPR off-target validation using target-specific DNA enrichment. *Nature communications, 11*(1), 1–11.

168. Schaefer, K. A., Wu, W.-H., Colgan, D. F., Tsang, S. H., Bassuk, A. G., & Mahajan, V. B. (2017). Unexpected mutations after CRISPR–Cas9 editing in vivo. *Nature methods, 14*(6), 547–548.

169. Ramirez-Phillips, A. C., & Liu, D. (2021). Therapeutic Genome Editing and In Vivo Delivery. *American Association of Pharmaceutical Scientists Journal, 23*(4), 80. https://doi.org/10.1208/s12248-021-00613-w

170. Singh, V., Braddick, D., & Dhar, P. K. (2017). Exploring the potential of genome editing CRISPR-Cas9 technology. *Gene, 599*, 1–18. https://doi.org/10.1016/j.gene.2016.11.008

171. Andhale, R., & Shrivastava, D. (2022). Huntington's disease: A clinical review. *Cureus, 14*(8), e28484. https://doi.org/10.7759/cureus.28484

172. Connor, A. A., & Gallinger, S. (2022). Pancreatic cancer evolution and heterogeneity: Integrating omics and clinical data. *Nature Reviews Cancer, 22*(3), 131–142. https://doi.org/10.1038/s41568-021-00418-1

173. Kudelova, E., Smolar, M., Holubekova, V., Hornakova, A., Dvorska, D., Lucansky, V., Koklesova, L., Kudela, E., & Kubatka, P. (2022). Genetic heterogeneity, tumor microenvironment and immunotherapy in triple-negative breast cancer. *International Journal of Molecular Science, 23*(23), 14937. https://doi.org/10.3390/ijms232314937

174. Pezeshkpoor, B., Oldenburg, J., & Pavlova, A. (2022). Insights into the Molecular Genetic of Hemophilia A and Hemophilia B: The Relevance of Genetic Testing in Routine Clinical Practice. *Hämostaseologie, 42*(6), 390–399. https://doi.org/10.1055/a-1945-9429

175. Hazafa, A., Mumtaz, M., Farooq, M. F., Bilal, S., Chaudhry, S. N., Firdous, M., Naeem, H., Ullah, M. O., Yameen, M., & Mukhtiar, M. S. (2020). CRISPR/Cas9: A powerful genome editing technique for the treatment of cancer cells with present challenges and future directions. *Life sciences, 263*, 118525.

176. Chira, S., Nutu, A., Isacescu, E., Bica, C., Pop, L., Ciocan, C., & Berindan-Neagoe, I. (2022). Genome editing approaches with CRISPR/Cas9 for cancer treatment: critical appraisal of preclinical and clinical utility, challenges, and future research. *Cells.* https://doi.org/10.3390/cells11182781

177. Gao, Q., Ouyang, W., Kang, B., Han, X., Xiong, Y., Ding, R., Li, Y., Wang, F., Huang, L., & Chen, L. (2020). Selective targeting of the oncogenic KRAS G12S mutant allele by CRISPR/Cas9 induces efficient tumor regression. *Theranostics, 10*(11), 5137.

178. Lentsch, E., Li, L., Pfeffer, S., Ekici, A. B., Taher, L., Pilarsky, C., & Grützmann, R. (2019). CRISPR/Cas9-mediated knock-out of krasG12D mutated pancreatic cancer cell lines. *International Journal of Molecular Sciences, 20*(22), 5706.

179. Miura, H., Quadros, R. M., Gurumurthy, C. B., & Ohtsuka, M. (2018). Easi-CRISPR for creating knock-in and conditional knockout mouse models using long ssDNA donors. *Nature protocols, 13*(1), 195–215.

180. Aregger, M., Xing, K., & Gonatopoulos-Pournatzis, T. (2021). Application of CHyMErA Cas9-Cas12a combinatorial genome-editing platform for genetic interaction mapping and gene fragment deletion screening. *Nature protocols, 16*(10), 4722–4765.

181. Gonatopoulos-Pournatzis, T., Aregger, M., Brown, K. R., Farhangmehr, S., Braunschweig, U., Ward, H. N., Ha, K. C., Weiss, A., Billmann, M., & Durbic, T. (2020). Genetic interaction mapping and exon-resolution functional genomics with a hybrid Cas9–Cas12a platform. *Nature biotechnology, 38*(5), 638–648.

182. Johansson-Åkhe, I., Mirabello, C., & Wallner, B. (2019). Predicting protein-peptide interaction sites using distant protein complexes as structural templates. *Scientific Reports, 9*(1), 1–13.

183. Chen, F., Alphonse, M., & Liu, Q. (2020). Strategies for nonviral nanoparticle-based delivery of CRISPR/Cas9 therapeutics. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 12*(3), e1609.

184. Mout, R., Ray, M., Lee, Y.-W., Scaletti, F., & Rotello, V. M. (2017). In vivo delivery of CRISPR/Cas9 for therapeutic gene editing: Progress and challenges. *Bioconjugate chemistry, 28*(4), 880–884.

185. van Haasteren, J., Li, J., Scheideler, O. J., Murthy, N., & Schaffer, D. V. (2020). The delivery challenge: Fulfilling the promise of therapeutic genome editing. *Nature biotechnology, 38*(7), 845–855.

186. Yip, B. H. (2020). Recent advances in CRISPR/Cas9 delivery strategies. *Biomolecules, 10*(6), 839.

187. Gao, X., Tao, Y., Lamas, V., Huang, M., Yeh, W.-H., Pan, B., Hu, Y.-J., Hu, J. H., Thompson, D. B., & Shu, Y. (2018). Treatment of autosomal dominant hearing loss by in vivo delivery of genome editing agents. *Nature, 553*(7687), 217–221.

188. Hansen-Bruhn, M., de Ávila, B. E. F., Beltrán-Gastélum, M., Zhao, J., Ramírez-Herrera, D. E., Angsantikul, P., Vesterager Gothelf, K., Zhang, L., & Wang, J. (2018). Active intracellular delivery of a Cas9/sgRNA complex using ultrasound-propelled nanomotors. *Angewandte Chemie International Edition, 57*(10), 2657–2661.

189. Ju, E., Li, T., Ramos da Silva, S., & Gao, S.-J. (2019). Gold nanocluster-mediated efficient delivery of Cas9 protein through pH-induced assembly-disassembly for inactivation of virus oncogenes. *ACS applied materials & interfaces, 11*(38), 34717–34724.

190. Yanez Arteta, M., Kjellman, T., Bartesaghi, S., Wallin, S., Wu, X., Kvist, A. J., Dabkowska, A., Székely, N., Radulescu, A., & Bergenholtz, J. (2018). Successful reprogramming of cellular protein production through mRNA delivered by functionalized lipid nanoparticles. *Proceedings of the National Academy of Sciences, 115*(15), E3351–E3360.

191. Zhou, W., Cui, H., Ying, L., & Yu, X. F. (2018). Enhanced cytosolic delivery and release of CRISPR/Cas9 by black phosphorus nanosheets for genome editing. *Angewandte Chemie, 130*(32), 10425–10429.

192. Shalaby, K., Aouida, M., & El-Agnaf, O. (2020). Tissue-specific delivery of CRISPR therapeutics: Strategies and mechanisms of

non-viral vectors. *International Journal of Molecular Sciences, 21*(19), 7353.

193. Rasul, M. F., Hussen, B. M., Salihi, A., Ismael, B. S., Jalal, P. J., Zanichelli, A., Jamali, E., Baniahmad, A., Ghafouri-Fard, S., Basiri, A., & Taheri, M. (2022). Strategies to overcome the main challenges of the use of CRISPR/Cas9 as a replacement for cancer therapy. *Molecular Cancer, 21*(1), 64. https://doi.org/10.1186/s12943-021-01487-4

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