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Review

The power and the promise of CRISPR/Cas9 genome editing for clinical application with gene therapy

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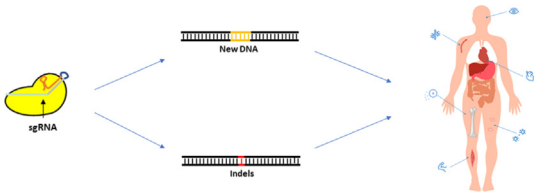
HIGHLIGHTS

- Due to its high accuracy and efficiency, CRISPR/Cas9 techniques may provide a great chance to treat some gene-related diseases.
- Researchers used the CRISPR/Cas9 technique to cure or alleviate cancers through different approaches, such as gene therapy and immune therapy.
- The treatment of ocular diseases by Cas9 has entered into clinical phases.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is derived from the bacterial innate immune system and engineered as a robust gene-editing tool. Due to the higher specificity and efficiency of CRISPR/Cas9, it has been widely applied to many genetic and non-genetic disease, including cancers, genetic hemolytic diseases, acquired immunodeficiency syndrome, cardiovascular diseases, ocular diseases, and neurodegenerative diseases, and some X-linked diseases. Furthermore, in terms of the therapeutic strategy of cancers, many researchers used the CRISPR/Cas9 technique to cure or alleviate cancers through different approaches, such as gene therapy and immune therapy.

Aim of Review: Here, we conclude the recent application and clinical trials of CRISPR/Cas9 in non-cancerous diseases and cancers and pointed out some of the problems to be solved.

Key Scientific Concepts of Review: CRISPR/Cas9, derived from the microbial innate immune system, is developed as a robust gene-editing tool and has been applied widely. Due to its high accuracy and efficiency, CRISPR/Cas9 techniques may provide a great chance to treat some gene-related diseases by disrupting, inserting, correcting, replacing, or blocking genes for clinical application with gene therapy.

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History of CRISPR

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) was first discovered in Escherichia coli and described as a 1,664-nucleotide sequence by Ishino and his colleagues in 1987 [1] (Fig. 1). Six years later, Mojica and Van Soolingen found similar DNA fragments in Haloarcula and Haloferax archaea[2], and M. tuberculosis[3] separately. By 2000, Mojica's group had identified

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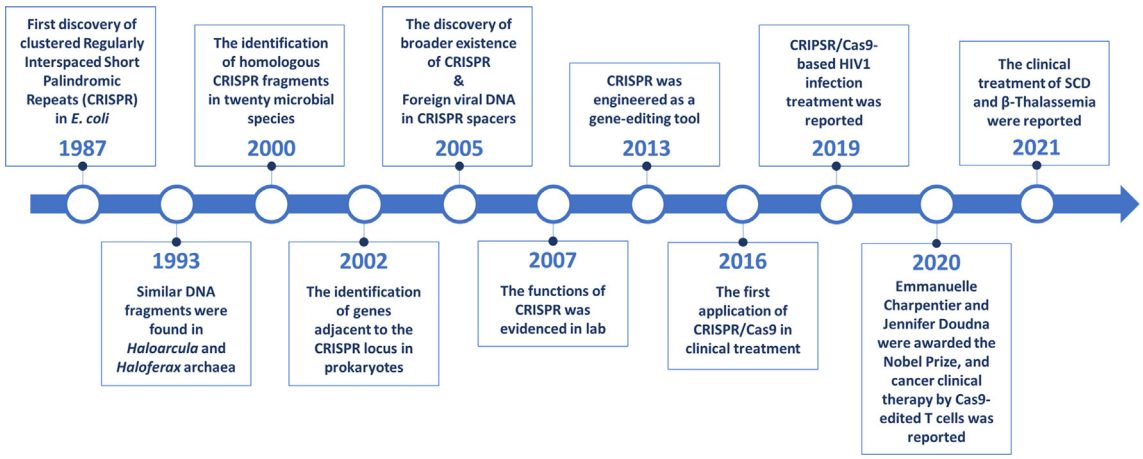

Fig. 1. The history of CRISPR techniques**.** CRISPR was firstly reported in E. coli in 1987, then was discovered widely from 1993 to 2005. After identification of genes adjacent to the CRISPR locus in 2002 and foreign viral DNA sequences in CRIPSR spacers in 2005, the functions of CRISPR was proven in 2007. In 2013, two labs simultaneously engineered CRISPR to become the most effective gene-editing tool available. The first CRISPR/Cas9 application in clinical treatment occurred in 2016 to treat lung cancer in China. In recent three years, several clinical treatments based on CRISPR/Cas9 techniques were reported. To recognize their contributions of CRISPR/Cas9 techniques, Emmanuelle Charpentier and Jennifer Doudna were awarded the Nobel Prize in 2020.

CRISPR DNA sequences in twenty microbial species[4]. Following the development of DNA sequencing techniques, the broader existence of CRISPR was reported in 2005: the majority of archaea (90%) and certain bacteria (40%) contain CRISPR fragments[5].

However, compared with its wide discovery in the first two decades, the functions of the CRISPR still kept unknown until 2007. Before making clear its functions, two crucial research speeded up the development: the study of genes adjacent to the CRISPR locus in 2002[6], and the discovery of foreign viral DNA in CRISPR spacers in 2005[5,7,8], which directly propelled the proposing of the hypothesis that the CRISPR system is an innate immune system in archaea and bacteria to fight against viruses[5,7,8]. Finally, in 2007, the hypothesis was verified by experiments in Barrangou's lab, and the functions of the 'weird' CRISPR sequences were finally figured out[9].

Due to the excellent ability to target and cleave specific DNA sequences, CRISPR was engineered as a gene-editing tool in 2012[10-13]. Compared with the previous gene-editing tools, including zinc finger nuclease (ZFN) and transcription activator-like effector nucleases (TALENs), the CRISPR-based gene-editing tool performs dramatically better. More importantly, CRISPR is easier to design for researchers, which accelerates its application widely. At present, CRISPR/Cas9 has been a routine and versatile method to edit genes over all the world. The first clinical application occurred in 2016. Chinese group injected CRISPR/Cas9-edited cells to treat aggressive lung cancer at the West China Hospital [14]. In recent years, a lot of clinical trials have been processing and some results have been reported, including the CRISPR/Cas9-based clinical treatment of acquired immunodeficiency syndrome (AIDS)[15], sickle cell disease (SCD)[16], β-thalassemia[16], and various cancers[17,18]. In recognition of their contribution to developing CRISPR/Cas9 genome editing techniques, Emmanuelle Charpentier and Jennifer Doudna were awarded the Nobel Prize in 2020 (Fig. 1).

Classification of CRISPR

The classification of CRISPR is updated continually. In 2015, only five types and 18 subtypes were recognized[19], while it had expanded to six types and 33 subtypes after five years[20]. According to the number of Cas proteins, CRISPR is generally divided into two classes: CRISPR/Cas system contains multiple Cas proteins in class 1 system, and only has a single Cas protein in class 2 system [21]. The type and subtype of CRISPR are based on different signature proteins. For example, Cas9 protein only exists in the type II CRISPR system, and Csn2 is the signature protein of subtype II-A. The class 1 CRISPR includes types I, III, and IV, whereas type II, V and VI belong to class 2 CRISPR[20]. More information about CRISPR classification has been concluded by Makarova[20].

Biosynthesis of CRISPR/Cas9

CRISPR/Cas9, the type II CRISPR system of class 2[20], is one of the most well-known CRISPR systems (Fig. 2). Gene-editing tool based on CRISPR/Cas9 is considered an efficient and reliable method to edit genes, and it is applied most widely among all CRISPR systems at present [21]. Intracellularly, genomic CRISPR/Cas9 locus comprises *trans*-activating CRISPR RNA (tracrRNA) gene, Cas9 gene, CRISPR repeat and spacer sequences, and their transcriptional products are tracrRNA, Cas9 protein, and Pre-crRNA separately[22]. After tracrRNA is anchored on the Cas9 enzyme, the dissociative part of tracrRNA is paired with the repeat sequences of Pre-crRNA to form Cas9 precursors. Finally, mature Cas9 complexes are formed by clipping Pre-crRNA under the catalyzation of RBase III[23].

Cas9 complexes in microbes could detect foreign DNA fragments complementary to spacer sequences then create double-strand breaks (DSBs) in the specific points, which provides innate protection against viral genomes. Based on its mechanism, Jinek's lab simplified and microbial CRISPR/Cas9 by fusing duo-tracrRNA:crRNA units as a single, truncated RNA chimera, and they utilized the engineered CRISPR/Cas9 to introduced DSBs successfully[10]. The simplified CRISPR/Cas9 only contains a single but bifunctional Cas9 protein[24] and a single small guide RNA (sgRNA) to catalyze both DNA targeting and cleaving processes, which promoted its wide use (Fig. 3A). Compared with the previous generation of gene-editing tools, including ZFN and TALENs, CRISPR/Cas9 is more specific but less cytotoxic[25,26]. It is also simpler to design for researchers, which speeds its applications up dramatically.

Mechanism of CRISPR/Cas9-mediated gene editing

Mechanically, CRISPR/Cas9 is quite different from the previous generation gene-editing tools. The previous tools, including ZFN

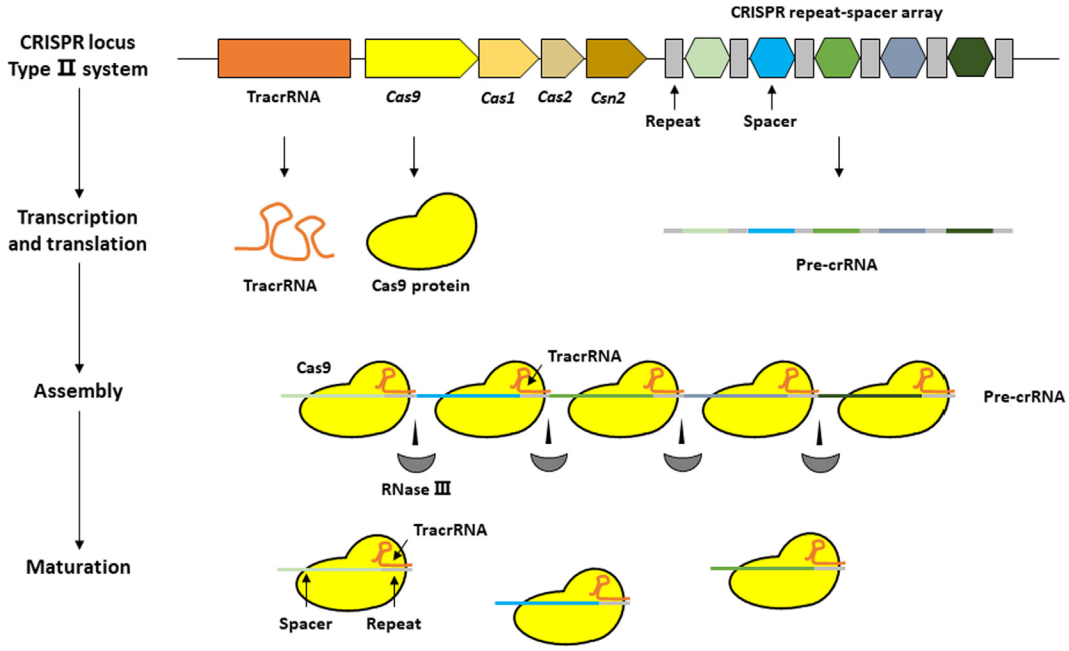

Fig. 2. Biosynthesis of CRISPR/Cas9. CRISPR locus mainly includes the DNA sequence of TracrRNA, CRISPR repeat-spacer array and Cas9 gene. Following transcription or translation, TracrRNA paired with the Pre-crRNA is anchored in the Cas9 protein, then the Cas9 complexes maturate after cleaving by RNase III.

and TALENs, are based on the pairing between protein domains and nucleotides. ZFN relies on multiple Cys2-His2 zinc-finger proteins to target specific NDA sequences. Each zinc-finger protein consists of about 30 amino acids and is capable of pairing three nucleotides. After designing and assembling different zinc-finger domains, ZFN can recognize the DNA sequences that contain 9–18 specific nucleotides[27]. The recognition domains of TALENs are derived from Xanthomonas, called transcription activator-like effector (TALE). Single TALE repeats are composed of 33–35 amino acids and recognize one nucleotide according to the 12th and 13th amino acids of TALE[28]. Both ZFN and TALENs exert cleavage functions by Fok I nuclease that is activated in dimers. Therefore, ZFN and TALENs need a pair respectively when editing genes, which increases the threshold and is inconvenient to design for reserachers[27,28].

CRISPR/Cas9 targets the specific DNA sequences via complementary base pairing. Engineered CRISPR/Cas9 gene-editing tools (abbreviated as CRISPR/Cas9 without specific explanation) consist of Cas9 nuclease and sgRNA (Fig. 3B). After binding between the Cas9-anchor sequence (tracrRNA or "scaffold" sequence) of sgRNA and the recognition lobe (REC) of Cas9, the user-defined 20 nucleotide targeting sequence (also called "spacer" sequence) in sgRNA targets the specific DNA site, then the Cas9 nuclease lobe (NUC) exert cleavage to generate DSB[29]. Intracellularly, DSBs are mainly repaired by two pathways: 1. non-homologous end-joining (NHEJ)[30], a pathway introduces random indels, leading to gene knocking out, and 2. homology-directed repair (HDR) [31], a pathway that needs homologic template sequences, resulting in gene correcting, knocking in or replacement. Noticeably, Protospacer Adjacent Motif (PAM), the first three bases in the spacer sequence, is essentially for gene targeting. CRISPR/Cas9 firstly binds with potential DNA fragments containing PAM sites. Then, the whole spacer sequence pairs the remanent nucleotides[32].

The further development of CRISPR/Cas9 technology

Following the rapid development and the considerable success of the CRISPR/Cas9 technique, CRISPR/Cas9 is re-engineered to apply in other aspects. For example, utilizing the targeting ability of CRISPR/Cas9, the CRISPR interference(CRISPRi) and catalytically-dead Cas9 enzyme (dCas9) were designed by removing nuclease domains to decrease or suppress gene expression [33] while Cas9 nickase (nCas9) that could create a gap in single DNA sequence only loss the partial functions of nuclease activity [34]. One year later after developing dCas9, the same lab fused transcription factors with dCas9 to amplify gene expression, namely CRISPR activation (CRISPRa)[35]. In addition, more accurate and safer CRISPR/Cas9-based gene-editing techniques without introducing DSBs, like base editing[36,37] and prime editing[38], were also developed. The base editing tools, including cytidine base editor (CBE) and adenine base editor (ABE), usually consist of cytidine deaminase/evolved adenine deaminase-fused nCas9/dCas9. The CBE realizes the conversion of C·G to T·A while the ABE enables the conversion of A·T to G·C, and the current developed nNme2-CBE system shows higher editing efficiency and flexibility[39]. However, the applications of base editing tools are still limited because they can only exert specific base conversion. Therefore, prime editing tools are developed via fusing engineered reverse transcriptase to dCas9 and adding primer sequences to gRNA (pegRNA) to realize the accurate gene editing with lower off-target effect[38]. Both base editing and primer editing tools are safer than the traditional CRISPR/Cas9 so they are potential to be applied in clinical gene therapy. Interestingly, Zhang F and his colleagues developed CRISPR-associated transposase in 2019, which also achieves gene insertion without DNA cleavage[40]. In terms of gene therapy in vivo, numerous Inducible CRISPR systems were studied, such as photoactivated and chemically induced Cas9 [41,42], and smaller-size CRISPR editing tools were also discovered recently[43].

Apart from that, the RNA-editing technique is also one of the most important aspects because RNA plays an essential role in gene expression and gene regulation. In 2014, Mitchell found CRISPR/Cas9 is possible to target RNA sequences rather than DNA after adding a PAM-presenting oligonucleotide (PAMmer)[44]. Based on Mitchell's research, Nelles and his colleagues utilized a fluorescent-protein-fused and nuclease-inactive Cas9, a modified

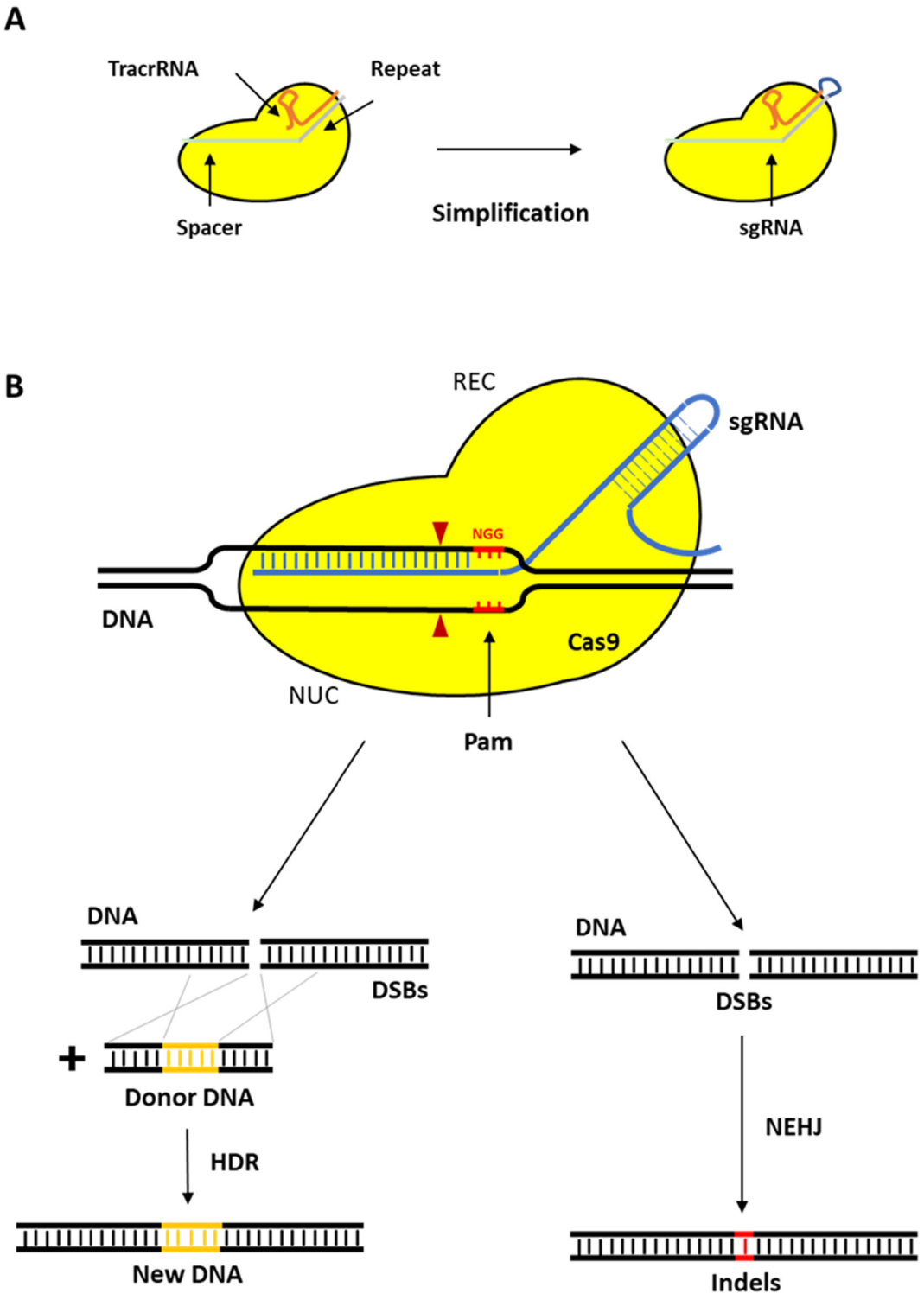

Fig. 3. A Simplification of CRISPR/Cas9. CRISPR/Cas9 is simplified as a robust gene-editing tool via fusing crRNA (spacer + repeat sequence) with TracrRNA as a single guide RNA (sgRNA). **B.** Mechanism of CRISPR/Cas9 gene-editing. Cas9 enzyme creates DSBs in the targeted DNA sequence. DSBs are repaired intracellularly via two ways: 1. HDR, DSBs are repaired with donor DNA to construct new DNA. 2. NEHJ, DSBs are repaired randomly, and random indels are introduced.

sgRNA, and a PAMmer to target mRNA sequences and realize intracellular transcript imaging in live cells, which provides a new method to visualize metabolism intracellularly[45]. Moreover, Abudayyeh's lab reported Cas13a (or C2C2) could target RNA sequences in 2016, suggesting CRISPR systems also protect microbes against RNA viruses[46], and Strutt redesigned CRISPR/Cas9 to target RNA two years later[47]. Finally, the discovery of CRISPR/Cas12a and the recently developed CHyMErA may overcome the challenges of multiple genome editing partly[48,49]. (Fig. 4, Table 1)

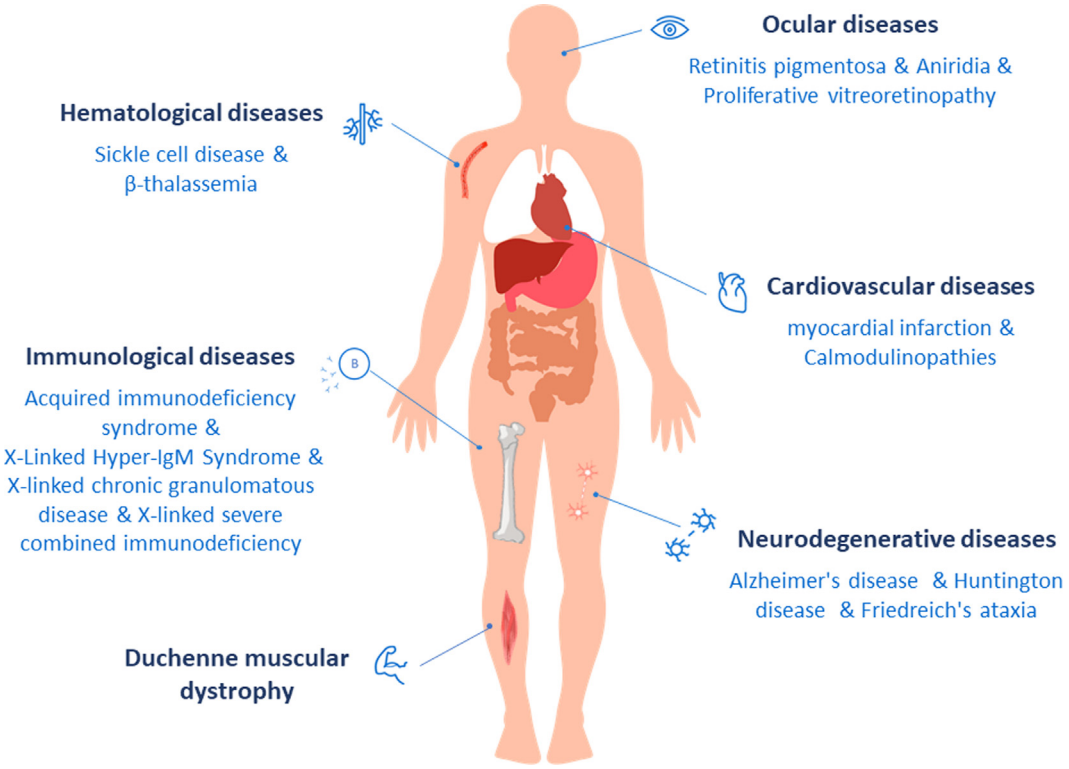

Fig. 4. Diseases that CRISPR/Cas9 is applied to. Several studied of non-cancerous diseases applied CRISPR/Cas9 techniques, and some of them have been possessing clinical stages.

Application of CRISPR/Cas9 in non-cancerous disease

Genetic hemolytic diseases

Sickle cell disease (SCD) is a typical and severe monogenetic disease due to β-globin (*HBB*) gene mutation, which causes the generation of abnormal HbS rather than the normal HbA. In the condition of low pressure of oxygen, HbS molecules interact with each other and form spiral-shaped polymers, leading to the sickle deformation of erythrocytes. The symptoms of SCD patients are unobvious in the initial three months after birth due to the presence of fetal hemoglobin (HbF), which is a kind of normal hemoglobin but is only expressed during the fetal period. Therefore, the strategies of SCD treatment mainly include correcting the β-globin gene to produce normal HbA and editing related regulatory genes to induce the reexpression of HbF.

In the aspect of the first strategy, Dever and DeWitt corrected the β-globin gene in patient-derived hematopoietic stem and progenitor cells (HSPCs) by CRISPR/Cas9 separately in 2016 and acquired great results in several weeks followed[50,51]. Two years later, the CRISPR/Cas9 delivery approach was optimized by Charlesworthr using adeno-associated virus (AAVs) with post-electroporation[52]. To reduce the off-target effect and increase editing efficiency, reengineered Cas9s were applied widely to edit genes in HSPCs. For example, Park utilized SpyCas9, a high-fidelity Cas9 enzyme with less off-target effect[53], to correct the β-globin gene, and Anzalone exerted gene-editing without DSBs by prime editing tool[38].

Except for direct β-globin gene correction, some research also edits related regulatory elements to relieve the patient's symptoms. As mentioned above, HbF, a normal but short-term-expressing hemoglobin, has the same functions as HbA and is considered another effective method to treat SCD. As described by Wu and Métais, the disruption of the BCL11A enhancer and HBG1/HBG2 gene promoter by CRISPR/Cas9 leads to HbF reexpression, which provides a novel therapeutic strategy for SCD[54,55]. Recently, the LRF-binding site of BCL11 was also identified as a potential therapeutic target of SCD[56]. Furthermore, the treatment of SCD by CRISPR/Cas9 has entered clinical phases, including one clinical survey (NCT03167450) and five clinical trials[16] (NCT03655678, NCT04819841, NCT04208529, NCT03745287, NCT04774536) (from clinicaltrials.gov, accessed 2021 -Jun-09).

Similar to SCD, β-thalassemia, another rare but severe monogenetic disease, is also mainly caused by the abnormal expression of β-globin. Therefore, the reexpression of HbF is a therapeutic strategy for β-thalassemia as well. To induce the production of γ-globin (one of HbF precursors), the 200 bp genomic region of the BCL11A enhancer was also knocked out[57], and 13 kb of the β-globin locus was detected in HSPCs via designing CRISPR systems for both upstream and downstream cleavage points[58], providing the experimental evidence for the application of CRISPR/Cas9 in β-thalassemia treatment. With the development of the related study, some research is nearing the clinical trial stage (NCT03655678, NCT03728322, NCT04925206, NCT04208529) (from clinicaltrials.gov, accessed 2021-Jun-09). Noticeably, the CRISPR/Cas9 based SCD/β-thalassemia CTX001 drug developed by Vertex Pharmaceuticals (NCT03655678, NCT03745287, and NCT04208529) has been applied to treat ten patients recently (seven with transfusion-dependent β-thalassemia and three with SCD). Despite some moderate side effects, the HbF levels of the initial two patients (one with transfusion-dependent β-thalassemia and one with SCD) are increased significantly in more than 99% pancellularity during a 12-month follow-up period[16].

X-linked diseases

Duchenne muscular dystrophy (DMD), one of the X-linked diseases, is caused by dystrophin gene frame-disrupting mutations.

Table 1
Clinical trials applying CRISPR/Cas9 for non-cancerous diseases (from clinicaltrials.gov, accessed 2021-Jun-09).

NCT Num.	Indications	Intervention/treatment	Status	Phase	Sponsor/ Collaborators	Start date	Results
Genetic hemolytic diseases							
NCT04774536	SCD	Drug: CRISPR_SCD001	Recruiting	1, 2	University of California, Los Angeles; University of California, Berkeley.	Dec/ 01/ 2021	No Results Posted
NCT03167450	SCD	Opinion survey	Completed	–	National Human Genome Research Institute (NHGRI)	Apr/ 28/ 2017	No Results Posted
NCT03655678	Beta-Thalassemia	Biological: CTX001	Recruiting	1, 2	Vertex Pharmaceuticals Incorporated,	Sep/ 14/ 2018	More than 99% pancellularity of two patients treated with CTX001 have dramatically increased HbF levels during 12 months. Adverse events include pneumonia in the presence of neutropenia, VOD–SOS, sepsis in the presence of neutropenia, cholelithiasis, and abdominal pain[16].
NCT03745287	SCD		Recruiting	1, 2	CRISPR Therapeutics	Nov/ 27/ 2018	
NCT04208529	Beta-Thalassemia; SCD.	Long-term Follow-up Study in Subjects Who Received CTX001	Enrolling by invitation	–	Vertex Pharmaceuticals Incorporated	Jan/ 25/ 2021	No Results Posted
NCT03728322	Thalassemia	Biological: iHSCs treatment group	Unknown	1	Allife Medical Science and Technology Co., Ltd.	Jan/ 01/ 2019	No Results Posted
NCT04925206	Beta-Thalassaemia	Biological: ET-01	Not yet recruiting	1	EdiGene (GuangZhou) Inc.	Aug/ 17/ 2021	No Results Posted
NCT04819841	SCD	Genetic: GPH101 Drug Product	Not yet recruiting	1, 2	Graphite Bio, Inc.	Sep/ 01/ 2021	No Results Posted
Acquired immunodeficiency syndrome							
NCT03164135	HIV-1-infection	Genetic: CCR5 gene modification	Unknown	Not Applicable	Peking University; Capital Medical University.	May/ 30/ 2017	During the 19-month follow-up period, 5.20 to 8.28% CRISPR-mediated CCR5 ablation efficiency was observed, and peripheral-blood CD4 + cells increased to the normal range with predictable side effects [15].
Ocular diseases							
NCT04560790	Viral Keratitis Herpes; Simplex Virus Infection.	Drug: BD111 Adult single group Dose	Active, not recruiting	1, 2	Eye & ENT Hospital of Fudan University	Nov/ 04/ 2020	No Results Posted
NCT03872479	Congenital Amaurosis 10	Drug: EDIT-101	Recruiting	1, 2	Editas Medicine, Inc.	Sep/ 26/ 2019	No Results Posted
Neurodegenerative diseases							
NCT03855631	Kabuki Syndrome 1	Genetic: Intervention on primary cultured cells	Completed	–	University Hospital, Montpellier; Association Française contre les Myopathies Telethon.	Sep/ 28/ 2020	No Results Posted
Others							
NCT04535648	Enterovirus Infections	Other: Non-invasive detection method: CRISPR techonology	Not yet recruiting	–	Children's Hospital of Fudan University	Oct/ 01/ 2021	No Results Posted
NCT04178382	Severe Sepsis	Diagnostic Test: PCR-CRISPR / Cas12a detection	Unknown	Not Applicable	Chinese Medical Association	Aug/ 01/ 2019	No Results Posted
NCT04074369	Tuberculosis	Diagnostic Test: CRISPR-based Test	Unknown	–	Huashan Hospital; Wenzhou Central Hospital; Hangzhou Red Cross Hospital.	May/ 01/ 2019	No Results Posted
NCT04535505	Pertussis	Diagnostic Test: Detection pathogenic pertussis by cross primer constant temperature amplification (CPA) and drug-resistant genes of erythromycin by CRISPR technology	Not yet recruiting	–	Children's Hospital of Fudan University	Jan/ 01/ 2022	No Results Posted

Table 1 (*continued*)

NCT Num.	Indications	Intervention/treatment	Status	Phase	Sponsor/ Collaborators	Start date	Results
NCT04990557	COVID-19 Respiratory Infection	Drug: PD-1 and ACE2 Knockout T Cells	Not yet recruiting	1, 2	Kafrelsheikh University	Aug/ 01/ 2021	No Results Posted
NCT03342547	Gastrointestinal Infection	Procedure: Duodenal biopsy Procedure: Saliva	Unknown	Not Applicable	Chinese University of Hong Kong	Apr/ 18/ 20118	No Results Posted
NCT04601051	Hereditary Transthyretin Amyloidosis	Biological: NTLA-2001	Recruiting	1	Intellia Therapeutics	Nov/ 05/ 2020	Patients treated by NTLA-2001 showed deceased serum transthyretin protein concentrations (mean reduction: 52 %) with mild adverse events[179].
NCT04478409	Familial Mediterranean Fever	Biological: one additional blood sample during a planned blood test	Not yet recruiting	–	Hospices Civils de Lyon	Nov/ 01/ 2020	No Results Posted
NCT04122742	Rubinstein-Taybi Syndrome	Biological: Culture of lymphoblastoid line from blood sample	Recruiting	–	University Hospital, Bordeaux	Oct/ 08/ 2019	No Results Posted

Dystrophin gene exists in x-chromosome and muatant *DMD* gene is recessive, so the percent of male DMD patients is significantly higher while the female are usually carriers without significant symptoms. To explore the effective therapeutic strategies of DMD, in 2016, Long and his colleagues utilized CRISPR/Cas9 to skip the mutated dystrophin exons in vivo via AAV vectors, which causes the expression of dystrophin protein[59]. Similar results were also acquired in the same year by Nelson and Tabebordbar after removing exon 23 of the dystrophin gene[60,61]. In later 2018, Ryu corrected the dystrophin gene precisely using base editing tools, which provides a safer method of DMD treatment[62].

Except for DMD, CRISPR/Cas9 techniques also provide a great chance to treat other severe X-linked diseases. For example, Kuo reported knocking in the cDNA of CD40 ligand by CRISPR/Cas9 could treat X-Linked Hyper-IgM Syndrome (XHIM)[63]. Moreover, the correction of the CYBB gene in HSPCs is one of the potential therapeutic strategies for X-linked chronic granulomatous disease (X-CGD)[64]. Recently, Pavel-Dinu's group demonstrated the effective therapy of X-linked severe combined immunodeficiency (SCID-X1) could be achieved by integrating a corresponding cDNA in long-term hematopoietic stem cells (LT-HSCs) using CRISPR/Cas9[65]. Similarly, Goodwin also edited the forkhead box protein 3 (FOXP3) gene by CRISPR/Cas9 for immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome treatment, which suggested gene correction by CRISPR/Cas9 is one of the most promising therapeutic approaches to genetic autoimmune diseases [66].

Acquired immunodeficiency syndrome

Acquired immunodeficiency syndrome (AIDS) is an untreatable disease caused by human immunodeficiency virus infection (HIV). The discovery and development of CRISPR/Cas9 techniques bring potential strategies against HIV infection. In the process of HIV infection, long terminal repeat (LTR) promotor plays a vital role in HIV gene expression, which is considered one of the therapeutic targets. Therefore, Ebina firstly edited the LTR promotor by CRISPR/Cas9 in 2013, causing the significant decrease of LTR-driven expression[67]. One year later, Hu and his colleagues also inactivated the HIV expression in infected microglial, promonocytic, and T cells by editing the LTR U3 region[68]. The recently developed CRISPR system was also applied for HIV treatment, such as CRISPR-Cas12a, a more active and specific CRISPR system. It was utilized by Gao to target and cleave the LTR sequence without pure DNA insertions[69]. Apart from LTR, the *pol* gene and *tat/rev* gene were also identified as therapeutic targets and inactivated by CRISPR/Cas9, inhibiting the expression of HIV genes to a different extent[70]. And Kaminski removed the whole HIV genome ex vivo to reduce the viral load in $CD4^+$ T cells[71]. Noticeably, in 2017, Yin's lab exerted CRISPR/Cas9-based editing of LTR and *Gag/Pol* gene in vivo by AAV delivery and reduced HIV RNA expression successfully, which promotes the development of Cas9-based HIV therapy significantly.

Moreover, gene editing of coreceptors is another approach to reducing or eliminate HIV infection except editing the HIV genome. As a typical example, CCR5 of human CD4 + cells was silenced by CRISPR/Cas9 in 2014, making $CD4^+$ cells acquire resistance against HIV[72]. After three years, Xu transplanted the CCR5-ablated $CD34^+$ HSPCs into mice, causing enrichment of $CD4^+$ T cells and a dramatic decrease in viral titration[73], and similar results were also acquired in 2019 using saCas9[74]. In addition, Liu also tried to edit coreceptors CXCR4 and CCR5 together in $CD4^+$ T cells by CRISPR/Cas9 using 'all in one' vector to reduce HIV infections[75]. Apart from that, Teng and his colleagues deleted miR-146a genomic sequences, whose transcriptional products suppress innate immune, to activate the immune system and limit HIV replication[76].

Following the development of CRISPR/Cas9 techniques and the comprehensive understanding of HIV, the potential of Cas9-based HIV therapy has been studied intensely. In early 2015, Zhu had detected ten possible targets of the HIV-1 genome for CRISPR/Cas9 and demonstrated the second exon (T10) of the *Rev* gene perform best among them[70]. In 2019, all of the gRNA and target sites for curing HIV-1 were reported by Sullivan, which facilitates further research into Cas9-based HIV-1 therapy[77]. Additionally, one clinical trial using CCR5 modified $CD34^+$ cells has been completed. The CRISPR/Cas9 based drug caused 5.20 to 8.28% CRISPR-mediated CCR5 ablation and the increased peripheral-blood CD4 + cell level with predictable side effects. But after interrupting antiretroviral therapy, the viral load goes up again, which indicates the effect of the drug is still limited(NCT03164135) (from clinicaltrials.gov, accessed 2021 -Jun-09)[15]. Thus, although CRISPR/Cas9 is a robust gene-editing tool, some problems in HIV application remain unsolved. As described by Wang in 2016, introducing indels in the HIV genome is lethal in most instances. Still, the survival viruses with replication ability will emerge the resistance to Cas9, which influences the effect of Cas-based HIV therapy[78].

Cardiovascular diseases

Cardiovascular diseases (CVDs) are the major cause of death globally, accounting for 32% of all deaths in 2019. According to

Ding's and Wang's reports, the destruction of the proprotein convertase subtilisin/kexin type 9 (PCSK9) gene by Cas9 in vivo reduced low-density lipoprotein cholesterol levels while strengthening the prevention of CVDs[79,80].

In 2018, Chadwick and his colleagues utilized CRISPR-engineered base editors to introduce nonsense mutations in vivo, resulting in a significant decrease of PCSK9 (about 50%) and plasma cholesterol level (about 30%)[81]. Recently, the knockdown of *PCSK9* by CRISPR-engineered base editors in primates achieved great success. After a single injection of lipid nanoparticles containing CRISPR modules, the level of PSK9 protein remains at only 10% of their original level for ten months, which may provide a convenient therapeutic approach for CVDs patients in the future[82]. Apart from *PCSK9*, *ANGPTL3* is also associated with CVDs. Therefore, Chadwick's lab reduced the level of low-density lipoprotein cholesterol and blood triglycerides by CRISPR-induced *ANGPTL3* mutations, which decreases the possibility of coronary heart disease[83].

In terms of myocardial infarction (MI), Cho integrated the *LEF1* gene into human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) then transplanted them into rat models, which increases the survival significantly[84]. In addition, a current study demonstrated that inhibiting the expression of the *calm2* gene by CRISPRi could treat Calmodulinopathies, a new kind of genetic arrhythmia syndrome, suggesting the potential clinical application of CRISPRi inhibitors[85].

Ocular diseases

CRISPR/Cas9 has been evidenced its potential to be applied in some genetic ocular diseases therapy, including cataracts, retinitis pigmentosa (RP), aniridia, and proliferative vitreoretinopathy (PVR). The earliest application of CRISPR/Cas9 in ocular diseases is to correct the *Crygc* gene in mouse to treat cataracts in 2013 [86]. Then it is widely used in RP treatment. For example, Bassuk repaired the GTPase regulator (*RPGR*) gene ex vivo[87], while Bakondi and Giannelli edited S334ter and P23H mutation of the *rhodopsin* (*RHO*) gene in vivo separately[88,89], to explore the therapeutic strategy of RP. And the last two studies in *RHO* results in the restoration of visual function and the slowdown of photoreceptor degeneration, suggesting CRISPR/Cas9 could be used in RP treatment[88,89].

Additionally, Mirjalili Mohanna successfully created a biological model for aniridia, then developed a strategy for its therapy by editing the *Sey* gene using CRISPR/Cas9[90]. Proliferative vitreoretinopathy (PVR) also benefits from the CRISPR/Cas9 techniques. As reported by Yang, deleting the gene of platelet-derived growth factor receptor β (*PDGFRβ*) in retinal pigment epithelial cells is a potential strategy for PVR treatment[91]. According to clinicaltrials.gov, two projects (NCT04560790 and NCT03872479) have been processed into clinical trials for ocular disease treatment (from clinicaltrials.gov, accessed 2021 -Jun-09).

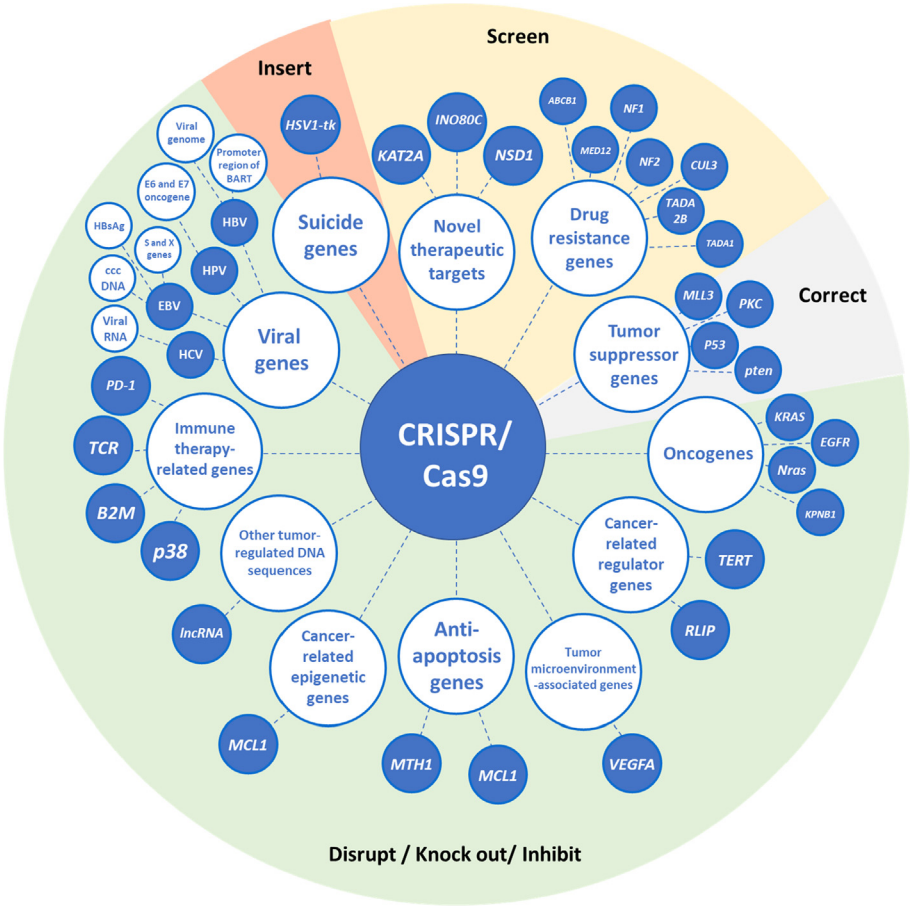

Fig. 5. Cancer targets that CRISPR/Cas9 is applied to**.** CRISPR/Cas9 is applied to screen cancerous novel therapeutic targets and drug resistance genes. And disrupting oncogenes or correcting tumor suppressor genes are routine and direct strategies to treat cancers. Furthermore, it is also effective to relieve cancer symptoms by editing cancer-related regulator genes, tumor microenvironment-associated genes, anti-apoptosis genes, cancer-related epigenetic genes and other related DNA sequences. Some studies also insert suicide genes to induce apoptosis of cancer cells. Moreover, CIRPSR/Cas9 is utilized to improve immunotherapy especially CAR-T therapy, and Cas9-based gene editing could prevent virus-induced cancers. Virus-induced cancers are potentially caused by HPV, HBV, HCV and EBV. Their effective targets are E6 and E7 oncogenes for HPV, HBsAg, cccDNA, S and X genes for HBV, viral RNA for HCV, and viral genome and promoter region of BART for EBV.

Neurodegenerative diseases

Neurodegenerative diseases (NDDs), such as Alzheimer's disease (AD) and Huntington disease (HD), results from the death or function loss of the neurocytes. For the application of CRISPR/Cas9 in AD, Wadhwani corrected the E4 allele of the apolipoprotein E gene (*APOE*) to the E3 isoform in stem-cell-derived neurons to decrease the phosphorylated tau production, which reduces the risk of AD significantly[92]. Moreover, CRISPR/Cas9 technique also provides a great chance to study the mechanism of AD further. For example, utilizing CRISPR/Cas9, Chiu demonstrated integrin-binding protein 1 (CIB1) might negatively regulate the production of amyloid β peptide, a protein associated with AD[93]. In addition, after deleting the *SORL1* gene, a selective effect on AD symptoms was observed, including endosome enlargement in human-induced pluripotent stem cells(hiPSC) neurons but not in hiPSC microglia, which further elucidated the pathogenesis of AD[94].

HD is another neurodegenerative disorder caused by CAG repeat expansion of the huntingtin gene (*HTT*). Therefore, Shin and Kolli inactivated the mutant allele to block the mutated HTT mRNA and protein generation in an in vitro model of HD[95,96], whereas Yang exerted Cas9-mediated suppression of endogenous mHTT in a mouse model[97]. Recently, six prevalent SNPs and all suitable PAM motifs of HTT exon-1 have been identified, facilitating further research on the treatment of HD[98]. Apart from that, CRISPR/Cas9 also provides a possible therapeutic strategy for Friedreich's ataxia (FRDA). For example, removing the GAA expansions of the frataxin gene (*FXN*) in vitro and in vivo alleviates related symptoms dramatically but with some unexpected side effects like p53-mediated cell proliferation delay[99]. At present, an increasing number of researchers pay more attention to NDDs research due to the apparent aging of the population in some countries. However, further studies are still needed for the more specific pathology and more effective Cas9-based treatment.(Fig. 5, Table 2)

Application of CRISPR/Cas9 in cancers

Cancer targets screening

Due to the capability of knocking genes out efficiently, CRISPR/Cas9 techniques developed as one of the most powerful tools to screen cancer targets. Compared with the screen approach via blocking gene expression by RNAi, CRISPR-mediated screen provides a strategy to efficiently identify more lethal genes and protein domains, causing a significant acceleration of cancer target discovery[100,101].

Benefiting from the development of the CRISPR technique, a number of novel therapeutic targets have been identified. For example, in 2015, Shi reported 19 unknown dependencies in acute myeloid leukemia cells (AMLCs) after targeting the exons of 192 chromatin regulatory domains by Cas9. And in the next two years, more therapeutic targets were discovered due to wider CRISPR-based genome screens, including *KAT2A* in AML and chromatin remodeling protein INO80C in mutant KRAS colorectal cancers [102,103]. Remarkably, a novel oncogene, namely *KPNB1*, was identified by Kodama in 2017, which provides a new therapeutic target for epithelial ovarian cancer (EOC)[104]. Currently, Zhang's lab utilized CRISPR/Cas9 to make clear the mechanism of *NSD1* and identified it as a novel target for human hepatocellular carcinoma (HCC)[105]. Furthermore, following the recent combination of computer data analysis and genetic screens, more efficient and comprehensive screen systems, such as CERES and DrugTargetSeqR[106,107], have been established. For instance, after analyzing the different dimensionalities of screen data, Behan demonstrated Werner syndrome ATP-dependent helicase is a lethal target in cancers[108], and Gurusamy identified p38 Kinase as an immunotherapeutic target[109].

In addition, the genetic screens based on CRISPR/Cas9 also reveals several genes related to the resistance of anti-cancer drugs. For example, for vemurafenib, an RAF inhibitor, Shalem utilized Cas9-mediated knockout screens to identify potential targets, including validated MED12 and NF1 genes as well as novel NF2, CUL3, TADA2B, and TADA1 genes[110]. Similarly, the mechanisms of ispinesib, YM155, selinexor, and topoisomerase II resistance were illustrated, providing the guidelines for further developing anti-cancer drugs[107,111,112]. Noticeably, Xu reported the depletion of the *ELP5* gene blocks U34 tRNA modification to inhibit the translation of hnRNPQ mRNA, which reveals *ELP5* plays a determinant role in gemcitabine sensitivity in gallbladder cancer[113].

The application of CRISPR/Cas9 provides a robust and efficient approach to screen cancer targets. However, there is no denying that some problems are present and remain unsolved. The high rate of false-positive results is the major one among them. As reported by Aguirre, the number of edited genes in cells is related to cell viability, which means the death or inaction of cells may be caused by the process of Cas9-mediated gene editing rather than the mutated genes[114]. To overcome the problem, except for the further development of computational correction and data analysis, the discovery or construction of more effective CRISPR/Cas9 delivery strategies that do not affect cell viability are also essential.

Cancer treatment

Traditionally, cancers are considered as the results of some mutated genes after being stimulated by carcinogens. Therefore, gene editing by CRISPR/Cas9 is a potential approach to fight against or even cure cancers. As a typical example, disrupting the most frequent mutated oncogene *KRAS* by Cas9 significantly reduces the viability of the cancer cells in vitro and the growth of tumors in vivo[115,116], and similar results were also acquired using CRISPRi to block *KRAS* expression[117]. Moreover, other oncogenes, such as the epidermal growth factor receptor (*EGFR*) gene in non-small cell lung cancer, were knocked out and achieved success in different degrees[118].

Apart from oncogenes, the mutation of tumor suppressor genes also plays an essential role in canceration. For instance, the mutation of mixed-lineage leukemia 3 (*MLL3*) gene promotes leukemogenesis[119], and the correction of the protein kinase C (*PKC*) gene suppresses tumor growth[120]. Furthermore, the disruption of the most well-known tumor suppressor genes *p53* and *pten* alone or in combination by Cas9 has been proved to significantly induce various cancers[121-123], which suggests correcting the tumor suppressor genes may reverse the cancerous or treat cancers[124].

In addition, as reported by Gao, the concurrent knock-in of oncogene *Nras* and disruption of suppressor gene *Pten* induced liver cancer, revealing that the oncogenes and tumor suppressor genes are the direct and major therapeutic targets for cancer treatment[125].

Viral infection is also one of the reasons for canceration. For example, it is well-known that human papillomaviruses (HPVs) infection results in cervical carcinoma, so the HPV vaccines are applied widely to prevent cervical carcinoma. However, vaccines are useless for patients who have been infected HPVs or suffer from cervical carcinoma, while CRISPR-mediated gene editing may provide a potential strategy to fight against HPVs. As reported by Hu and Kennedy, CRISPR/Cas9 could disrupt HPV oncogenes E6 and E7 in vitro, causing infected cell cycle arrest and apoptosis [126,127]. The in vivo experiment also evidenced the accessibility of cervical carcinoma treatment via targeting HPV E6 and E7 by

Table 2
Clinical trials applying CRISPR/Cas9 for cancers (from clinicaltrials.gov, accessed 2021-Jun-09).

NCT Num.	Indications	Intervention/treatment	Status	Phase	Sponsor/ Collaborators	Start date	Results
NCT03057912	Human Papillomavirus-Related Malignant Neoplasm	Biological: TALEN Biological: CRISPR/Cas9.	Unknown	1	First Affiliated Hospital, Sun Yat-Sen University; Jingchu University of Technology.	Jan/ 15/ 2018	No Results Posted
NCT04426669	Gastrointestinal Epithelial Cancer; Gastrointestinal Neoplasms; Cancer of Gastrointestinal Tract; Gastrointestinal Cancer; Colo-rectal Cancer; Pancreatic Cancer; Gall Bladder Cancer; Colon Cancer; Esophageal Cancer; Stomach cancer.	Drug: Cyclophosphamide; Drug: Fludarabine; Biological: Tumor-Infiltrating Lymphocytes (TIL); Drug: Aldesleukin.	Recruiting	1, 2	Intima Bioscience, Inc.; Masonic Cancer Center, University of Minnesota.	May/ 15/ 2020	No Results Posted
NCT03399448	Multiple Myeloma; Melanoma; Synovial Sarcoma; Myxoid/Round Cell Liposarcoma.	Biological: NY-ESO-1 redirected autologous T cells with CRISPR edited endogenous TCR and PD-1; Drug: Cyclophosphamide; Drug: Fludarabine; Device: NY-ESO-1 expression testing.	Terminated	1	University of Pennsylvania; Parker Institute for Cancer Immunotherapy; Tmunity Therapeutics.	Sep/ 05/ 2018	CRISPR/Cas9 exerts gene editing in human-derived T cells with 40% single mutation, 20% double mutation, and 10% tribble mutation. Injecting CRISPR-engineered T cells shows long-term persistence and high-level engraftment[17].
NCT03545815	Solid Tumor	Biological: anti-mesothelin CAR-T cells	Recruiting	1	Chinese PLA General Hospital	Mar/ 19/ 2018	No Results Posted
NCT04037566	Acute Leukemia Lymphocytic	Genetic: XYF19 CAR-T cell; Drug: Cyclophosphamide; Drug: Fludarabine.	Recruiting	1	Xijing Hospital	Aug/ 01/ 2019	No Results Posted
NCT04244656	Multiple Myeloma	Biological: CTX120	Recruiting	1	CRISPR Therapeutics AG	Jan/ 22/ 2020	No Results Posted
NCT04438083	Renal Cell Carcinoma	Biological: CTX130	Recruiting	1	CRISPR Therapeutics AG	Jun/ 16/ 2020	No Results Posted
NCT04502446	T Cell Lymphoma	Biological: CTX130	Recruiting	1	CRISPR Therapeutics AG	Jul/ 31/ 2020	No Results Posted
NCT04035434	B-cell Malignancy; Non-Hodgkin Lymphoma; B-cell Lymphoma; Adult B Cell ALL.	Biological: CTX110	Recruiting	1	CRISPR Therapeutics AG	Jul/ 22/ 2019	No Results Posted
NCT04637763	Relapsed Non-Hodgkin Lymphoma; Refractory B-Cell Non-Hodgkin Lymphoma; B Cell Non-Hodgkin's Lymphoma.	Genetic: CB-010; Drug: Cyclophosphamide; Drug: Fludarabine.	Recruiting	1	Caribou Biosciences, Inc.	May/ 26/ 2021	No Results Posted
NCT04557436	B Acute Lymphoblastic Leukemia	Drug: PBLTT52CAR19	Recruiting	1	Great Ormond Street Hospital for Children NHS Foundation Trust; University College, London.	Aug/ 12/ 2020	No Results Posted
NCT03747965	Solid Tumor	Biological: Mesothelin-directed CAR-T cells	Unknown	1	Chinese PLA General Hospital	Nov/ 01/ 2018	No Results Posted
NCT03166878	B Cell Leukemia; B Cell Lymphoma.	Biological: UCART019	Recruiting	1, 2	Chinese PLA General Hospital	Jun/ 01/ 2017	No Results Posted
NCT03606486	High-Grade Ovarian Serous Adenocarcinoma	Other: Biospecimen Collection; Other: Laboratory Biomarker Analysis; Device: Lavage; Other: Pap Smear.	Recruiting	Not Applicable	University of Washington; Minnesota Ovarian Cancer Alliance.	Nov/ 16/ 2018	No Results Posted
NCT03081715	Esophageal Cancer	Other: PD-1 Knockout T Cells	Completed	Not Applicable	Hangzhou Cancer Hospital; Anhui Kedgene Biotechnology Co., Ltd.	Mar/ 14/ 2017	No Results Posted
NCT03332030	Neurofibromatosis Type 1; Tumors of the Central Nervous System.	Diagnostic Test: Collection of Stem Cells	Suspended	–	Roger Packer	Nov/ 27/ 2015	No Results Posted

Table 2 (*continued*)

NCT Num.	Indications	Intervention/treatment	Status	Phase	Sponsor/ Collaborators	Start date	Results
NCT03398967	B Cell Leukemia B Cell Lymphoma	Biological: Universal Dual Specificity CD19 and CD20 or CD22 CAR-T Cells	Recruiting	1, 2	Chinese PLA General Hospital	Jan/ 02/ 2018	No Results Posted
NCT04976218	Advanced Biliary Tract Cancer	Biological: TGFβR-KO CAR-EGFR T Cells	Not yet recruiting	1	Chinese PLA General Hospital	Aug/ 01/ 2021	No Results Posted
NCT02793856	Metastatic Non-small Cell Lung Cancer	Drug: Cyclophosphamide; Other: PD-1 Knockout T Cells.	Completed	1	Sichuan University; Chengdu MedGenCell, Co., Ltd.	Aug/ 26/ 2016	After infusion, CRISPR/Cas9-engineered T-cells were observed in peripheral blood with 7.7-week median progression-free survival and 42.6-week median overall survival. The median off-target rate was 0.05%, and adverse events were grade ½[18].
NCT04767308	CD5 + Hematopoietic Malignancies; Chronic Lymphocytic Leukemia; Mantle Cell Lymphoma; Diffuse Large B-cell Lymphoma; Follicular Lymphoma; Peripheral T-cell Lymphomas.	Biological: CT125A cells; Drug: Cyclophosphamide, fludarabine.	Not yet recruiting	1	Huazhong University of Science and Technology; Shanghai IASO Biotechnology Co., Ltd.	Mar/ 01/ 2021	No Results Posted
NCT04417764	Advanced Hepatocellular Carcinoma	Procedure: Transcatheter arterial chemoembolization; Biological: PD-1 knockout engineered T cells	Recruiting	1	Central South University	Jun/ 20/ 2019	No Results Posted
NCT02863913	Invasive Bladder Cancer Stage IV	Biological: PD-1 Knockout T Cells; Drug: Cyclophosphamide; Drug: IL-2.	Withdrawn	1	Peking University	Sep/ 01/ 2016	No Results Posted
NCT02867345	Hormone Refractory Prostate Cancer	Biological: PD-1 Knockout T Cells; Drug: Cyclophosphamide; Drug: IL-2.	Withdrawn	1	Peking University	Nov/ 01/ 2016	No Results Posted
NCT02867332	Metastatic Renal Cell Carcinoma	Biological: PD-1 Knockout T Cells; Drug: Cyclophosphamide; Drug: IL-2.	Withdrawn	1	Peking University	Nov/ 01/ 2016	No Results Posted
NCT03044743	Stage IV Gastric Carcinoma Stage IV Nasopharyngeal Carcinoma T-Cell Lymphoma Stage IV Stage IV Adult Hodgkin Lymphoma Stage IV Diffuse Large B-Cell Lymphoma	Drug: Fludarabine; Drug: Cyclophosphamide; Drug: Interleukin-2.	Recruiting	1, 2	The Affiliated Nanjing Drum Tower Hospital of Nanjing University Medical School	Apr/ 07/ 2017	No Results Posted

Cas9. After introducing indels in E6 and E7, the dramatical inhibition of tumorigenesis and tumor growth was observed[128], and the treatment combining with *cis*-diamine-dichloro platinum II (CDDP)[129].

Moreover, hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are also risky, causing almost 80% of hepatocellular carcinoma (HCC) in the world. According to previous studies, high HBV surface antigen (HBsAg) is highly related to HBV-associated HCC development, so Zhen and Song knocked out the HBsAg gene to inhibit HBV expression and replication[130,131]. Remarkably, Song clarified the pathway of HBsAg in HCC development in 2018, which ensures the anti-cancer effect of disrupting the HBsAg gene theoretically[131]. Apart from HBsAg, utilizing CRISPR/Cas9, Seeger disrupted covalently closed circular DNA (cccDNA) to inhibit HPV infections up to eightfold[132], whereas Zhu targeted HBV S and X genes to produce an anti-cancer effect[133]. Different from HBV, the genetic information of HCV is RNA rather than DNA. Therefore, Price utilized the Cas9 enzyme from Francisella novicida (FnCas9) that is capable of targeting RNA directly to inhibit viral RNA, causing a significant decrease in HCV protein production [134]. Additionally, an increasing number of studies pay more attention to the Epstein-Barr virus (EBV) because EBV infection is highly related to many malignancies. Some research reported that a dramatic inhibition of EBV, including proliferation arrest and viral load decrease, was observed after targeting the viral genome or promoter region of BamHI A rightward transcript (BART) through CRISPR/Cas9[135-137], which reduces the risk of canceration effectively. Although the infection of these viruses does not mean cancers absolutely, decreasing or removing viral load is a robust approach to prevent or treat cancers, and CRISPR/Cas9 is one of the robust tools to achieve that.

Except for genes that cause cancers directly, some genes that promote tumor growth or prevent apoptosis of tumor cells are also identified as treatment targets for cancers. For example, vascular endothelial growth factor A (VEGFA) is a tumor microenvironment-associated gene highly expressed in osteosarcoma (OS), while CD44 is highly related to tumor progression and metastasis, and their Cas9-mediated disruptions inhibit OS and metastasis significantly[138,139]. The *DNMT1* gene was targeted by CRISPR/Cas9 due to the high level of aberrant methylation in tumor cells, causing the slowing down of tumor growth in vivo [140]. Then in recent two years, Li used CRISPR-engineered base

editor to edit mutated TERT promoters to suppress brain tumor growth[141], and Singhal demonstrated knocking Ral-interacting protein (RLIP) gene out relieves the effect of the *p53* mutation [142]. Noticeably, long non-coding RNA (lncRNA) has been proved as an up regulator in bladder cancer. After targeting lncRNA by CRISPR/Cas9, the proliferation, migration, and invasion of tumors were suppressed dramatically[143]. Apart from tumor regulator genes, some researchers also disrupted anti-apoptosis genes, such as MTH1 and MCL1, to induce the apoptosis of cancer cells [144,145]. Interestingly, Chen harnessed CRISPR/Cas9 to insert herpes simplex virus type 1 thymidine kinase (HSV1-tk) sequence, a suicide gene, causing the decrease of tumor size successfully, which suggests suicide-gene insertion by Cas9 may be a potential approach to treat cancers[146].

In recent years, immune therapy, especially CAR-T therapy, has been considered one of the most promising approaches to treat cancers. Generating allogeneic universal CAR-T cells from healthy donors is one of the most feasible and durable strategies[147]. Fully allogeneic CAR-T cells need to knock out TCR and HLA and transduce CAR simultaneously, so flexible and efficient CRISPR/Cas9 is widely applied in CAR-T studies and production[148]. Moreover, some genes like *PD-1* prevent the tumor cells from the immune system by controlling the apoptosis of immune cells. Therefore, some researchers disrupted *PD-1* by CRISPR/Cas9 to enhance the activity and cytotoxicity against tumor cells [149,150]. And the anti-cancer effect of CAR-T therapy is improved significantly in vivo after targeting endogenous TCR, β-2 microglobulin (B2M), and *PD1* in combination or simultaneously, providing some solutions for the underachieving CAR-T therapy [151]. Moreover, drug resistance is still a problem for cancer therapy though some resistance genes have been screened. Except for those genes described before, Liu and his group reported inhibiting the *ABCB1* gene could revert multi-drug resistance (MDR) to doxorubicin, revealing CRISPR/Cas9-mediated gene editing is a potential approach to solve MDR effectively[152].

At present, more than 30 clinical trials that apply CRISPR techniques to treat cancers are processing, and some have achieved partial success (from clinicaltrials.gov, accessed 2021 -Jun-09). For example, the first application of CRISPR/Cas9 occupied in 2016 to treat lung cancer[14]. Moreover, recent CRISPR/Cas9 engineered T cells have excellent performance: a clinical trial (NCT03399448) that utilized CRISPR/Cas9 to remove *PD-1* and *TCR* shows long-term persistence and high-level engraftment with a mild side effect[17], another one (NCT02793856) that only edited *PD-1* reports CRISPR-engineered T cells are generally safe and feasible, which suggests the potential applications of CRISPR/Cas9-based cancer therapy[18]. In summary, more recent clinical trials focus on immune therapy, especially CAR-T therapy rather than gene therapy because gene editing is exerted ex, which avoids a lot of problems such as immune response. Following the development of CRISPR techniques and a better understanding of cancers, more effective and efficient cancer therapy strategies will process into clinical trials and find the final answers for cancers in the future.

Problems of CRISPR/Cas9

Delivery challenge

Although CRISPR/Cas9 is a mature gene editing technology and has been used widely, therapeutic CRISPR/cas9 maintains many problems due to the off-target effect, low efficiency, and packaging challenges. In terms of CRISPR-based gene therapy, the difficulties of the delivery system in vivo are highlighted mainly[153]. An ideal delivery method for therapeutic CRISPR/cas9 should have the features of high delivery efficiency, great targeting ability, and ease of mass production. However, the current strategies are still far from reaching the ideal bay[154].

Physical strategies of CRISPR/Cas9 delivery are usually applied in vitro or ex vivo but rare in vivo. Except for traditional electroporation and microinjection, more efficient methods, including ultrasound-propelled nanomotors[155], microfluidic or nanofluidic approaches[156], and lance assay nanoinjection[157] are also utilized to deliver CRISPR systems. But for the CRISPR/Cas9 mediated gene-editing in human primary immune cells, electroporation is still the first choice in several studies[48]. Interestingly, some physical approaches is able to deliver CRISPR/Cas9 in vivo. For example, hydrodynamic injection (HDI) was reported as a novel approach to CRISPR system delivery[158], but its application is limited to a few organs, such as the liver, because the method may cause damage during delivery.

Apart from physical strategy, researchers also deliver CRISPR/Cas9 via different vectors. According to the types of vectors, the method of CRISPR/Cas9 delivery could be divided into two kinds: viral strategies and non-viral strategies. Considering the moreextraordinary delivery and targeting ability, viruses are usually utilized and engineered to deliver CRISPR/Cas9. Adeno-associated virus (AAV) is the commonest vector for gene therapy in vivo and ex vivo due to its wide serotype, little immunogenicity, and toxicity[159], but the small payload (only 4.5–5 kb) limits its development. Compared with AAV, lentivirus (LV) and adenovirus (AdV) have a better capacity and allow delivery of additional genetic compounds, such as multiple promotors. Thereinto, one of the biggest advantages of AdV is its ability to transfer a wider range of cells than LV and AAV. However, the bigger sizes of LV and AdV could trigger strong humoral and even cellular immune responses, which suggests low efficiency of delivery and potential risk of inflammation[154]. Other viral vectors are also applied recently and have their own characteristics respectively. For example, EBV vectors are able to express exogenous genes more stably [160], and Sendai viral vectors are capable of infecting broader host types[161], while Baculovirus vectors have a bigger payload[162]. However, these vectors are only used ex vivo by now, but they are still potential to be applied in vivo in the future after optimizing.

-Regarding non-viral strategies, liposomes are utilized most frequently because they have been merchandised widely, and some researchers also try to use gold nanoparticles (AuNPs) as the vectors for CRISPR/Cas9 delivery. Other non-viral delivery vectors, including Lipofectamine RNAiMAX[163], PolyJet™ In Vitro DNA Transfection Reagent[126], and X-tremeGENE HP DNA Transfection Reagent[126], are also commercial but are only suitable for in vitro or ex vivo experiments by now. In terms of in vivo delivery, thousands of studies focus on discovering and synthesizing high-efficient and low-cytotoxic non-viral vectors. Present strategies include common nano-sized preparations (e.g., self-assembled micelles[164] and polyethylene glycol phospholipid-modified cationic lipid nanoparticle[165] for CRISPR/Cas9 plasmid delivery, and DNA nanoclews[166] and black phosphorus nanosheets[167] for CRISPR/Cas9 complex delivery), receptor-mediated delivery strategies (e.g., folate receptor-targeted liposomes deliver CRISPR/Cas9 plasmids[140]), cell-penetrating peptides (CPPs)-mediated delivery strategies (e.g., Kim fused Cas9 protein with a low molecular-weight protamine and a nuclear localization sequence to deliver CRISPR/Cas9 complex[168], while Wang established modified cationic α-helical polypeptides based PEGylated nanoparticles to deliver CRISPR/Cas9 plasmids and sgRNA[169]), and multi-model delivery strategies (e.g., R8-dGR peptide modified cationic liposome for the delivery of CRISPR/Cas9 and sgRNA plasmids[170], and near-infrared upconversion–activated system for CRISPR/Cas9 complex delivery[171]). Still, a number of troubles in the field of non-viral strategies, like delivery barriers or endo-

some evasion, remain unresolved, which stagnates the further development of therapeutic CRISPR/cas9[154].

In addition to various delivery vectors, the forms of cargos also play an important role in CRISPR/Cas9 delivery. Traditionally, Cas9 is delivered in the form of DNA or mRNA with sgRNA and template sequence together. In order to increase gene editing efficiency, Yin and his colleagues delivered Cas9 mRNA by lipid nanoparticles while delivering sgRNA and template sequence by AAV separately[172]. Moreover, Cas9 proteins can also be delivered into cells directly by fusion or recruitment methods. It avoids the risk of genome integration and reduces the off-target effect due to the short half-life of Cas9 protein, which is considered a safer approach for gene therapy[173]. In conclusion, there is an opposite problem in CRISPR delivery challenges, including the smaller size of the delivery vectors to avoid immune response but the bigger requirement of cargo loads to carry more CRISPR or expressive modules. Therefore, how to balance these two problems and develop an optimized delivery strategy are the aspects that need to be studied further.

Off-target effect

The off-target effect is one of the CRISPR/Cas9 application limitations and is considered a significant risk factor during gene therapy in vivo. Although several computer software has optimized the design of sgRNA, its specificity cannot be ensured absolutely. Furthermore, except for sgRNA design, the duration of the Cas9 enzyme also plays an important role. Therefore, disposable Cas9 design, such as delivering protein directly, could significantly decrease the off-target effect. In addition, optimized Cas9 enzyme is also an approach to reduce the off-target effect, including SpCas9-HF1[174] and eSpCas9[175]. However, the off-target effect in vivo keeps unsolved, and it is also highly related to the delivery strategy.

Pam limitation

As described above, the Pam sequence is essential for CRISPR/Cas9 targeting, and only the DNA sequences that contain Pam could be targeted by the Cas9 enzyme. However, Pam limits the design of sgRNA and decreases the flexibility of CRISPR/Cas9 significantly. Although an increasing number of CRISPR types are discovered, causing more Pam fragments are selectable at present. The compulsory Pam insertion still affects the design of sgRNA in some situations. Therefore, how to develop a designable Pam is significant to broaden the application of CRISPR/Cas9.

Immune response

As a foreign protein, Cas9 may induce the immune response. Although there are not many reports about the severe immune reaction caused by Cas9, the antibodies of Cas9 have been widely identified in human bodies[176], which suggests the potential risk of inflammation during CRISPR/Cas9-based gene therapy. At present, researchers pay more attention to the immunogenicity caused by delivery vectors, especially the viral vectors, because the human body may have been infected by these viruses before and contain the corresponding antibodies already. Collectively, the immune reaction potentially induced by CRISPR/Cas9 gene-editing system is one of the major risk factors in the development of CRISPR-based gene therapy in vivo.

Multiple gene-editing

CRISPR/Cas9 is an efficient gene-editing tool but only edits one gene with a sgRNA simultaneously. Therefore, multiple gene-editing utilizing CRISPR/Cas9 have to rely on various sgRNAs, which decreases the editing efficiency while increases the delivery difficulty. Recent CRISPR/Cas12a may overcome the challenge, but other subsequent problems are still unsolved, including inactivation of cells and cell cycle arrest after multiple gene-editing. In the future, effective multiple gene-editing strategies will dramatically promote the gene therapy of polygenic diseases and cancers.

Outlook

CRISPR/Cas9, derived from the microbial innate immune system, is developed as a robust gene-editing tool and has been applied widely. Due to its high accuracy and efficiency, CRISPR/Cas9 techniques may provide a great chance to treat some gene-related diseases by disrupting, inserting, correcting, replacing, or blocking genes.

Cas9-mediated gene editing has been utilized to treat various non-cancerous diseases. Monogenetic diseases and X-linked diseases caused by gene mutation are the most direct and apparent kinds that CRISPR/Cas9 can be applied to. A number of studies have proved gene correction in monogenetic diseases and X-linked diseases is an effective therapeutic strategy, and several related clinical trials have been in the process recently. Similarly, the risk of CVDs is reduced, and the symptom of NDDs is relieved dramatically after targeting related genes using CRISPR/Cas9. At the same time, the treatment of ocular diseases by Cas9 has entered into clinical phases. Noticeably, AIDS may become curable through knocking out the viral genes by Cas9, which benefits millions of patients in the world.

In terms of cancer therapy, CRISPR/Cas9 was initially applied in drug targets screen, causing a rapid discovery of lots of novel drug targets. Combined with computer and data techniques, Cas9-based target screening provides an advanced approach to understand cancers better. Disrupting oncogene or correcting tumor suppressor genes alone or in combination are the major strategy to treat cancers while knocking out viral genomes like HPV decreases the risk of virus-induced tumors. Moreover, some researches also demonstrate some regulator genes, epigenetic genes, and microenvironmental genes also play vital roles in cancerization and are developed as effective therapeutic targets. Recently, more researchers focus on the immune therapy of cancers. Especially CAR-T therapy has been applied in clinical treatment and achieves success to some extent, and the therapeutic effect may be improved by inhibiting some related genes by Cas9.

Admittedly, CRISPR/Cas9 is a robust gene-editing tool. However, some problems keep unsolved, including off-target effect, delivery challenges, PAM limitation, and immunogenicity, which blocks its application in clinical therapy. When developing gene therapy, the off-target effect and editing efficiency are two of the most concerning problems because the off-target effect may cause unexpected editing of normal genes and then lead to severe diseases or even death, while editing efficiency directly affects the therapeutic effect. To solve these two problems, lots of studies work in different aspects. Firstly, the delivery system of CRISPR/Cas9 is critical for CRISPR-based treatment. For example, the delivery efficiency determines the efficiency of Cas9-mediated gene editing to a considerable extent, and the targetability, stability, and release time of delivery vectors are highly related to the off-target effect. As mentioned above, disposable designs (such as delivering Cas9 protein) and all-in-one designs (such as delivering Cas9 plasmids and sgRNA simultaneously) are effective ways to decrease the off-target effect. Secondly, re-engineered or optimized Cas9 proteins reduce the off-target effect as well. Compared with traditional CRISPR/Cas9-based gene editing, base editing and primer editing tools do not create DSBs when editing genes, which dramatically

decreases the off-target effect. And their editing efficiency is continually enhanced in recent studies via optimizing enzymes or pegRNA, suggesting the great potential for clinical application. Finally, sgRNA design is still essential because it plays a key role in gene targeting. Except for optimizing sgRNA design rules and computer programs, the studies to avoid PAM limitation may improve the specification and flexibility of sgRNA, which is beneficial to improve editing efficiency while reducing the possibility of off-target. At present, most clinical trials just limit in editing genes in patient-derived cells ex vivo, and then the cells are injected back into the patient's bodies, such as the treatment of SCDs and immune therapy (Fig. 6). This method avoids the risk of off-target effect and delivery challenge but is not suitable for all diseases. And recent research prefers to disrupt or knock out genes rather than correcting because the additional DNA templates increase the delivery difficulty. The clinical application of CRISPR/Cas9 is still at an early stage, and the prioritized problems of clinical gene therapy by Cas9 in vivo are off-target effect and delivery challenges.

Compared with monogenetic diseases, Cas9-based gene therapy of cancers are more challenging due to multiple gene mutations. Although it is available to exert multiple gene editing by CRISPR/Cas9 after adding the corresponding sgRNAs. CRISPR/Cas9-mediated multiple gene editing is not widely applied in clinical treatment or even gene function studies because it could lead to several potential problems, such as severe off-target effect and the deletion of big DNA fragments[177]. Therefore, novel approaches in multiple gene editing need to be developed further to overcome the present challenges.

Except for CRISPR/Cas9, other CRISPR systems, including Cas12a, Cas3 (with Cascade), Cas13, dCas9, and nCas9, also contain huge potentials for gene therapy[178]. For example, similar to Cas9, Cas12a also belongs to the class II CRISPR system. But Cas12a generates a staggered cut rather than the flat end that Cas9 creates, which is a great advantage when integrating DNA sequences. In the Cas3 system, the Cascade complex binds and recognizes the target DNA sequence then Cas3 proteins are recruited to generate a single-strand nick. Due to the promiscuous recognition of PAM in the Cas3 system, it is more flexible to target specific DNA sequences than Cas9. Different from Cas9, Cas12a, and Cas3 systems, Cas13 is an RNA-guided RNA targeting system. Cas13 could edit single-strand RNA efficiently, while nuclease-inactive dCas13 is able to regulate protein translation. Both dCas9 and nCas9 lose the nuclease activity but maintain the ability to target DNA sequences, so a lot of re-engineered CRISPR/Cas9 tools, such as CRISPRi, CRIPARa, base editing tool, and primer editing tools, etc., are based on dCas9 or nCas9.

In conclusion, CRISPR/Cas9 is an efficient gene-editing tool but not a perfect treatment approach at present. Lots of problems need to be developed further until its reliability and safety maintain a higher level. Cell therapy by Cas9 seems to be more simple to design while avoiding some troubles that in vivo gene therapy meets. However, only a few of hundreds of diseases could be treated by cell therapy. To provide a broader therapeutic strategy for genetic diseases, gene therapy by Cas9 is one of the major aspects to develop in the future. Therefore, how to efficiently and safely edit genes by CRISPR/Cas9 in vivo will be listed at the top in the next decade.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

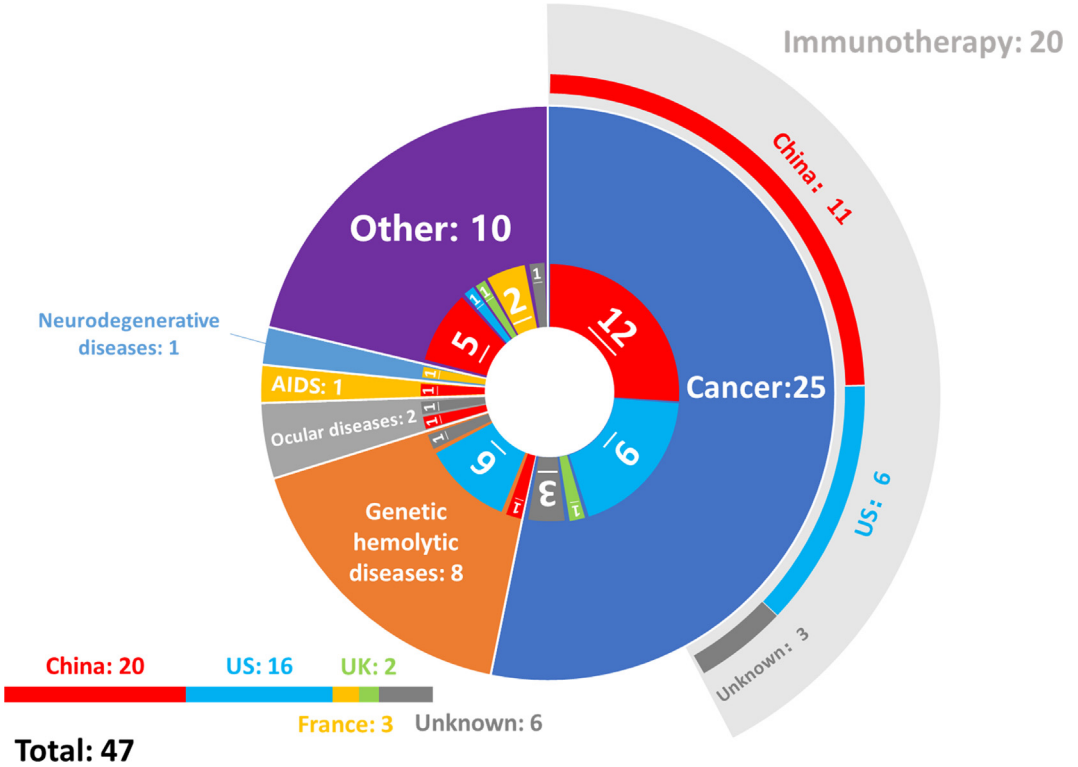

Fig. 6. Clinical trail distribution. The recent CRISPR/Cas9-based clinical trails focus on the cancer and genetic hemolytic diseases treatment, accounting for 25 and 8 of 47 respectively, and 20 of 25 clinical projects adopts the immunotherapy for cancer treatment. China and US dominates the recent CRISPR/Cas9-based clinical trails, accounting for 20 and 16 of 47 respectively (from clinicaltrials.gov, accessed Jun-09–2021).

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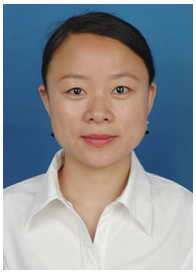

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