Advancements and Obstacles of CRISPR-Cas9 Technology in Translational Research

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The expanding CRISPR-Cas9 technology is an easily accessible, programmable, and precise gene-editing tool with numerous applications, most notably in biomedical research. Together with advancements in genome and transcriptome sequencing in the era of metadata, genomic engineering with CRISPR-Cas9 meets the developmental requirements of precision medicine, and clinical tests using CRISPR-Cas9 are now possible. This review summarizes developments and established preclinical applications of CRISPR-Cas9 technology, along with its current challenges, and highlights future applications in translational research.

Gene therapy is a form of disease treatment that involves the alteration of DNA or RNA, thereby counteracting or remedying the conditions caused by malfunctioning genes.[1] Although gene therapy remains a risky technique and is being tested, it is undoubtedly a promising treatment option for human diseases,[2–5] including inherited disorders, some types of cancer, and certain viral infections, because it has the capacity to cure diseases at the roots.[1] Such nucleic acid manipulation techniques must rely on rapid developments in deep genome and transcriptome sequencing platforms that allow precise and comprehensive analysis of disease pathogenesis. Disease treatment is inevitably focused on determining unique molecular characteristics of individual patients, as a subfield of precision medicine.[6] Among the tools available for precision-based gene therapy, CRISPR-Cas9 has revolutionized the field, allowing for simple, time-saving, and cost-efficient eukaryotic genome editing.[7] Since 2013, when this technology was validated in human cells, the RNA-guided CRISPR-Cas9 system has been applied for DNA and RNA manipulation in numerous cell lines and organisms.[8,9] CRISPR-Cas9 can generate loss-of-function mutations in genes, precise genome-site knockins, site modifications, and transcriptome and epigenome alteration.[10] Considering its expanding repertoire of applications, CRISPR-Cas9 is a promising method for optimizing biomedical research and innovate treatment strategies for gene-associated diseases such as hereditary diseases[11] and malignancies.[12] Thus far, CRISPR-Cas9 has successfully cured genetic diseases in animal models. The first clinical trials involving CRISPR-Cas9 were initiated in 2016 and continue to this day.[13] Excitingly, a recently approved clinical trial (ClinicalTrials.gov: NCT03655678) will use CRISPR-Cas9-modified therapeutic human hemopoietic stem cells (hHSCs), called CTX001, to treat thalassemia.

Unlike in genetic diseases, one or two causative genes cannot explain cancer pathogenesis. Because of the complex biological characteristics of cancers, the search for an optimal treatment for cancer patients has been an ongoing race. The most promising tumor treatment is immunotherapy, involving the use of immune checkpoint inhibitors and adoptive immune cell therapy. CRISPR-Cas9 technology is able to transfer tumor-specific identification signals into cytotoxic immune cells and remove immune repressor genes of these cells that are retransfused into patients for cancer treatment. The therapeutic potential is being investigated in multiple clinical trials (ClinicalTrials.gov: NCT02793856, NCT03044743, NCT03398967, and NCT03081715).[13]

This review summarizes the basic mechanisms and general applications of CRISPR-Cas9 gene editing and discusses technological applications, advancements, and challenges of this exciting technology in preclinical and clinical translational research.

Overview of CRISPR-Cas9 Technology

CRISPR-Cas9 technology was first described as a series of short repeats interspaced with short sequences in the *Escherichia coli* genome[14] and was officially named in 2002.[15] The technology was functionally verified as adaptive immunity[16] and used to cleave target DNA sequences.[17] Because this technology was initially used to target the human genome in 2013,[18–20] its applications have increased tremendously in biomedical research (Figure 1). Based on effector proteins, CRISPR-Cas systems are grouped into two classes that are further subdivided into six types (I–VI) in accordance with their signature genes (class 1: types I, III, and IV; class 2: types II, V, and VI).[21] Class 2 systems are characterized by single-subunit effector proteins such as Cas9 and Cas12a (Cpf1). These have been more successfully reprogrammed for genome engineering than class 1 systems, although the latter comprise ~90% of all CRISPR-Cas systems identified in bacteria and archaea.[22,23]
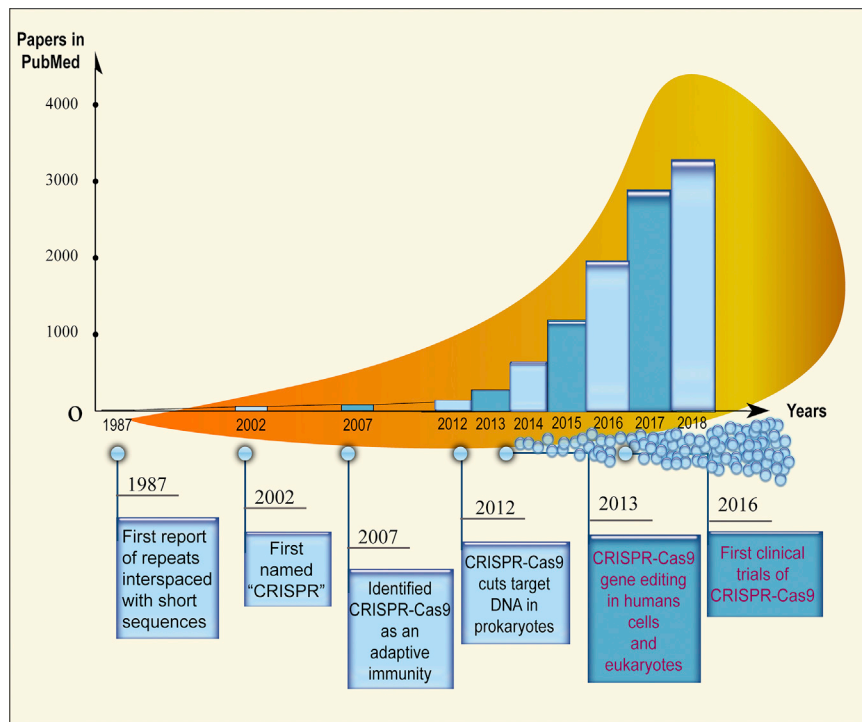
Figure 1. Timeline and Key Studies of the CRISPR-Cas System

Major developments during the past three decades are shown. Research on CRISPR exploded after 2013 (represented by ever-increasing number of bubbles), when the technology was used to modify genes in human cells and many other eukaryotes.

Compared with conventional gene-editing tools (zinc-finger nucleases [ZFNs] and transcription activator-like effector nucleases [TALENs]), CRISPR-Cas9 is easier and faster, because the system requires only one Cas9 endonuclease and a short single gRNA.[7] The following sections describe the most important applications of CRISPR-Cas9 thus far. First, the system generates animal and cellular models of diseases more rapidly and conveniently[28] and provides novel insights into disease treatment.[29] Such methods include rectification of genomic variations in human embryonic cells and stem cells.[30] Second, high-throughput CRISPR can help screen candidates during systematic genetic functional analyses and identify new drug targets.[24,31] Third, dCas9 is useful for regulating gene expression[32] and for posttranscriptional RNA base editing.[9,33] Fourth, CRISPR-Cas9 is valuable for nucleic acid-based imaging, detection, and diagnosis.[10] Finally, Figure 3 summarizes the applications of CRISPR-Cas9 technology in agriculture, material science, and various other fields.[33]

Genome engineering using CRISPR-Cas9 is initiated with RNA-guided Cas9 endonuclease inducing double-strand breaks (DSBs) adjacent to PAM (a short, Cas9-recognized sequence) in the genome of transformed cells. These breaks then activate DNA repair mechanisms through two possible pathways. Target gene functional knockout occurs upon triggering of the non-homologous end joining (NHEJ) pathway. The second pathway is homology-directed repair (HDR), which introduces a precise genetic knockin through an exogenous template[7,24] (Figure 2A).

Other than these basic manipulations, advancements in CRISPR-Cas9 technology have resulted in extended complex applications. The system can perform pooled high-throughput genetic screens (Figure 2F).[24] In addition, dead Cas9 (dCas9) is a catalytically deactivated Cas9 nuclease (Figure 2C) that can be fused with transcription factors, fluorescent marker proteins, and other functional proteins to regulate gene expression, track target genes, and make unexpected genomic alterations.[7] Fusion of a deaminase to dCas9 generated a novel base-editing technology (Figure 2D), resulting in precise and highly efficient C-to-T or G-to-A modifications.[25] Moreover, dead guide RNA (dgRNA), with a short 14- to 15-nucleotide (nt) sequence and MS2-binding loops into the backbone, can activate transcription without inducing DSBs (Figure 2E).[26] Furthermore, the development of double-nickase Cas9 (Figure 2B) expands the translational application of CRISPR-Cas9 through the generation of a staggered single cleavage in the genome, reducing off-target effects.[7] This method minimizes off-target editing and has been used in multiple studies.[27]

Major Advancements in the Application of CRISPR-Cas9 in Genetic Diseases

In non-human studies, CRISPR-Cas9 is routinely used for genomic and transcriptomic engineering to induce epigenetic effects.[10,29,34] While these non-patient experiments provide a fundamental platform for understanding the complex action mode of CRISPR-Cas9 components, we focus on studies that have greater direct clinical translation.

To date, only studies on cells and small animals have provided support for the therapeutic efficiency of employing CRISPR-Cas9 gene-editing technology to rectify pathological mutations that causing genetic diseases. Successful examples include gene rectification in Duchenne muscular dystrophy (DMD),[35–38] sickle cell disease (SCD),[39,40] β-thalassemia,[41–43] and hereditary tyrosinemia type I (HT1).[44,45] Specifically for DMD, some data regarding the efficacy and safety of CRISPR-Cas9 gene editing are available from studies on large animals. The use of adeno-associated viruses for intramuscular delivery of CRISPR-Cas9 gene-editing components to dogs with DMD improved the histological features of muscle tissue and partially restored dystrophin expression several weeks later.[46] Although such findings represent a crucial step of this technology
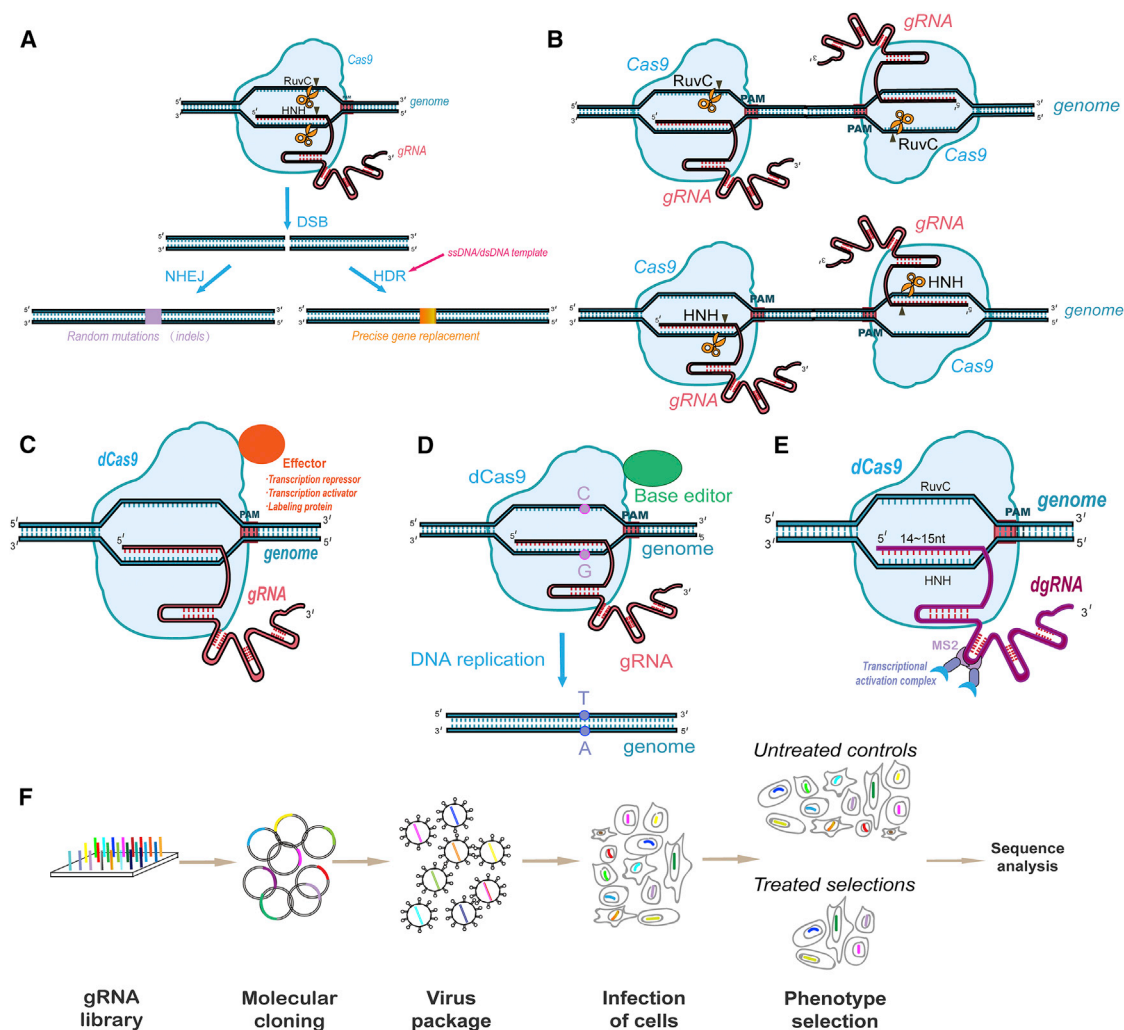
Figure 2. Mechanisms of CRISPR-Cas9 as a Genome Engineering Platform

(A) Cas9 nuclease cleaves double-stranded DNA via RuvC and HNH domains to introduce double-strand breaks (DSBs) that are then repaired by NHEJ or HDR. Error-prone NHEJ repair pathways always introduce random insertions or deletions (indels) (left), but with the use an exogenous DNA donor, the HDR pathway can introduce precise insertions (right). (B) Cas9 nickase (nCas9) can cleave a single strand of double-stranded DNA when inactivating either HNH or RuvC domains. The use of two nCas9 complexes can reduce off-target effects. (C) Dead Cas9 (dCas9) contains inactivating domains in HNH or RuvC. It can be tethered with transcriptional factors to mediate downregulation or activation of target genes. In addition, dCas9 can be fused to labeling proteins (e.g., GFP), for nucleic acid imaging. (D) dCas9 can be fused to deaminase for catalytic conversion of C to U, thus achieving single-base editing during DNA replication. Similarly, dCas9 can be tethered with epigenetic modification enzymes to obtain desired edits. (E) dgRNA can guide Cas9 to regulate gene expression. (F) Workflow schematic of CRISPR genome-scale functional screening.

toward clinical translation, its long-term efficiency and safety in patients must still be considered.

Although existing treatments can attenuate the symptoms of some hereditary diseases, patients still suffer from considerable psychological or social pressure for possessing mutation-associated conditions. Although considerable ethical and regulatory issues remain to be addressed, major progress has been made with the use of CRISPR-Cas9 technology to rectify pathogenic gene mutations in human embryos.[47] The CRISPR-Cas9 base editor (BE) has been reported to yield targeting efficiency of 89% in rectifying *FBN1* mutations in Marfan syndrome[30] but only 23% in rectifying *HBB-28* (A > G) mutations in β-thalassemia.[48] More encouragingly, CRISPR-Cas9 gene editing has successfully helped rectify *MYBPC3* mutations associated with hypertrophic cardiomyopathy in human embryos, with a targeting efficiency of 72.2%, and embryos exhibited no mosaicism, no off-target gene editing, and no other abnormalities.[49] Moreover, embryos preferred the gene copies of the healthy parent to that of an exogenous DNA donor as a repair template. Such a preference may reduce ethical concerns to a certain extent, although further studies are required to improve the targeting efficiency and determine the long-term safety of CRISPR-Cas9 technology. A study using
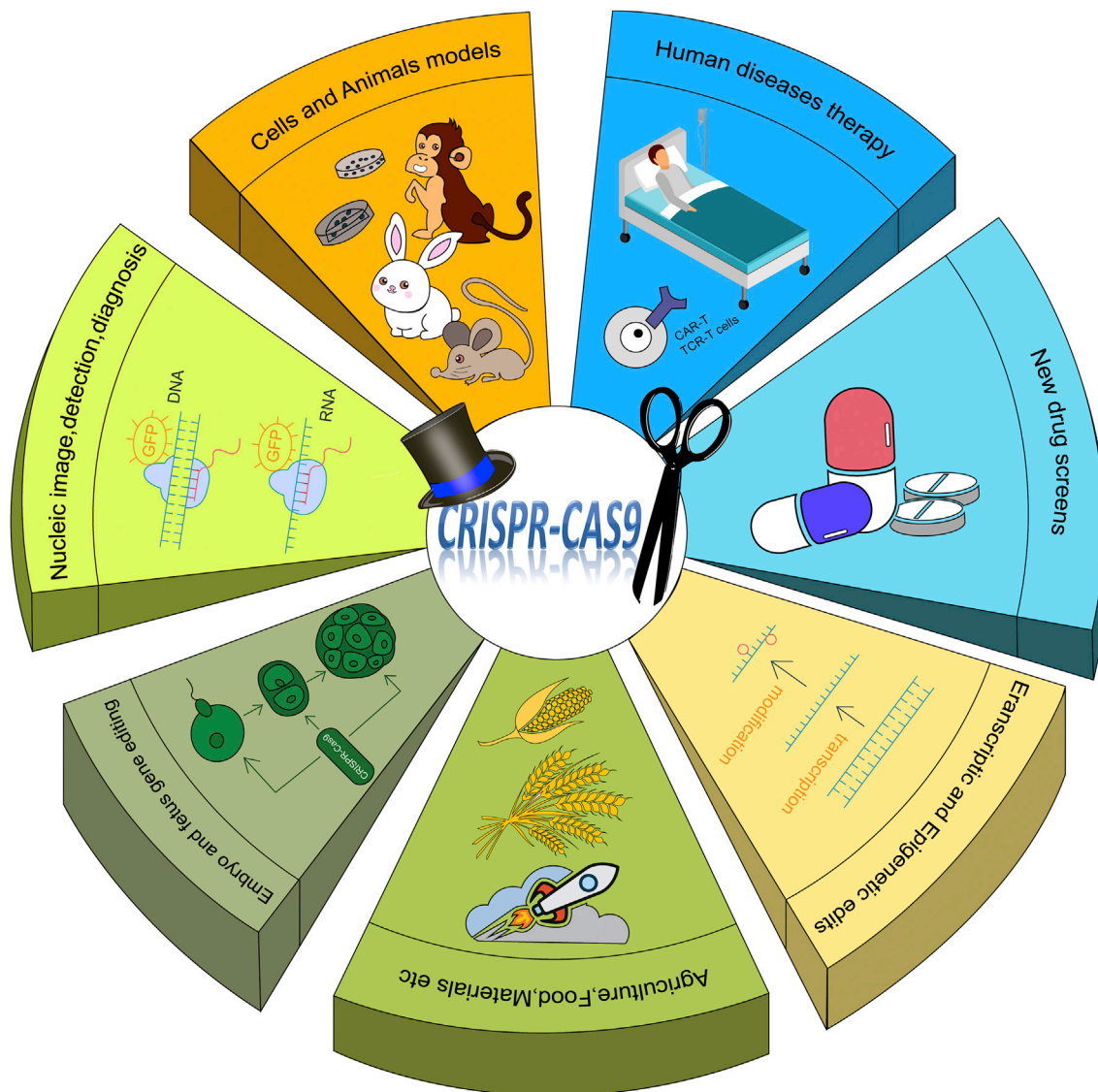
Figure 3. Applications of CRISPR-Cas9 Genomic Engineering

As a genetic and epigenetic engineering technology, CRISPR-Cas9 has a range of applications. This study focuses on biomedical and clinical applications.

CRISPR-Cas9 BE to edit murine pathological *Pcsk9* and *Hpd* genes *in utero* reported high editing efficiency, tolerance to CRISPR components, and amelioration of murine HT1 at birth even without nitisinone treatment.[50] These studies have strong clinical implications for CRISPR-Cas9 gene editing in human embryos and fetuses.

Significant advancements have been made in using CRISPR-Cas9 to treat other diseases on some genetic basis. For example, transcriptionally activated CRISPR-Cas9 with short dgRNA (~14 or ~15 bp) successfully alleviated disease phenotypes in mouse models of type 1 diabetes, acute kidney injury, and muscular dystrophy.[51] Similarly, CRISPR-mediated genome editing partially improved symptoms in SOD1-linked forms of amyotrophic lateral sclerosis and autosomal

dominant hearing loss.[52,53] These advancements provide promising insights into the eventual translation of CRISPR gene editing into the clinical setting.

Promising Breakthroughs in Cancer Treatment Using CRISPR-Cas9 Technology

CRISPR-Cas9 genome editing has been considered a powerful tool in cancer research. The technology can be used to investigate tumorigenesis and tumor invasion.[54] Encouraging studies suggest that CRISPR-Cas9 is valuable for the rapid development of cancer immunotherapy.

Adoptive gene-engineered T cell immunotherapy is one of the most promising cancer treatment strategies, including chimeric antigen

receptor (CAR)- and T cell receptor (TCR)-T cell therapy. The former has succeeded in treating hematological malignancies, but not in treating solid tumors.[55] CARs generally comprise an extracellular tumor antigen-recognized domain and an intracellular signal-activating domain.[56] The primary contribution of CRISPR-Cas9 technology is to simplify CARs or TCR gene editing, thus elevating the potency and safety of cancer-adoptive T cell therapy. For example, using CRISPR-Cas9 to direct CD19-specific CAR sequences to the T cell receptor α constant (TRAC) locus reportedly reduced T cell exhaustion and enhanced CAR-T cell therapeutic potency in a NOD scid gamma (NSG) mouse model of acute lymphoblastic leukemia.[57] In addition, CRISPR-Cas9-transfected human TCR-T cells showed anti-tumor reactivity both in melanoma cell lines and in NSG mouse models of human melanoma.[58] Therapeutic T cells engineered through endogenous TCR modification with CRISPR-Cas9 and exogenous TCR gene transfer were more sensitive to hematological malignancies *in vitro* than standard TCR-transduced T cells.[59]

Extensive applications of CAR-T cell therapy in various tumors is deterred because existing CARs are unspecific and do not strictly recognize cancer antigens over normal tissues.[60] Fortunately, CRISPR technology can be employed to identify novel CARs that can potentially address this issue. Pooled high-throughput CRISPR screening of tumor models can help identify novel therapeutic targets for immunotherapy, such as *PTPN2* in melanoma mouse models.[61,62] Moreover, CRISPR-Cas9 technology has been used to develop CD33 knockout hematopoietic stem cells that allowed anti-CD33 CAR-T cells to specifically eliminate acute myeloid leukemia (AML) tumor cells without affecting normal myeloid cells.[60] Another concern in adoptive immunotherapy is the possibility of triggering graft-versus-host disease. Although this problem can be avoided by using autogenous T cells, it is time consuming and costly to produce sufficient therapeutic cells tailored to individual patients.[56] Using CRISPR-Cas9 technology to edit CAR-T cells would help overcome immunological incompatibility and decrease expenses, because the edited non-alloreactive cells are usable in any patients. For example, CRISPR-Cas9 gene editing was reportedly used to generate allogeneic CAR-T cells deficient in TCR and human leukocyte antigen (HLA) class I molecules. The experiments demonstrated that these CAR-T cells showed anti-tumor activity in the mouse model, which was enhanced by the simultaneous knockout of programmed cell death 1 (PDCD1 or PD1).[63] Gene-edited CAR-T cells did not induce graft-versus-host disease, thereby greatly increasing the practicality of allogeneic T cells in immunotherapy.

Successful tumor inhibition involves improvement of CAR and TCR specificity and obliteration of tumor immune escape. Immune-suppressive pathways in tumor microenvironment include PD1/PDL1, CTLA-4/B7-2, and TIM3/Galectin-9.[64,65] Inhibition of the PD1/PDL1 pathway using systemic PD1 monoclonal antibodies (e.g., ipilimumab, pembrolizumab, and nivolumab) constitutes a promising cancer treatment approach.[66] However, systemic administration of PD1 inhibitors could cause immune-related side effects.[67] Adoptive T cell immunotherapy using CRISPR-Cas9 to generate PD1-disrup-

ted human primary T cells partially avoided those side effects even as it enhanced cytokine secretion and toxicity against tumor cells both *in vitro* and *in vivo*.[63,68,69] This promising outcome eventually led to the first clinical trial that investigated the safety and efficacy of CRISPR-Cas9-engineered PD1-knockout T cells in lung cancer patients.[13] Ultimately, the combination of CAR modification, immune checkpoint inhibition, and CRISPR-Cas9 technology is a therapeutic strategy with massive potential for advancements in treating solid tumors.[63,69]

Clinical Trials

This section summarizes the details of all clinical trials with CRISPR-Cas9 technology regardless of status and disease types (Table 1), although none of them have posted definite results. Multiple clinical trials have been launched to investigate the safety and efficiency of CRISPR-Cas9 technology-mediated, PD1-knockout, engineered T cells on lung cancer patients (ClinicalTrials.gov: NCT02793856) and esophageal cancer patients (ClinicalTrials.gov: NCT03081715). In addition, PD1-knockout Epstein-Barr virus (EBV)-cytotoxic T lymphocytes were being tested in treating advanced-stage EBV-associated malignancies (ClinicalTrials.gov: NCT03044743). The investigators extracted patients' T lymphocytes, performed *ex vivo* PD1 knocked out with CRISPR-Cas9 technology, and then selected, expanded, and infused engineered T cells back into patients who had been pretreated with drugs like cyclophosphamide and hydrocortisone before infusion. Treatment-emergent adverse events and tumor response efficiency evaluation were recorded and analyzed. Peripheral blood samples were also collected to monitor some parameters, including interleukins, interferons, tumor necrosis factor, and circulating tumor DNA. Similar clinical trials in which PD1-knockout T cells are used to treat other types of tumor, including bladder cancer (ClinicalTrials.gov: NCT02863913), prostate cancer (ClinicalTrials.gov: NCT02867345), and renal cell carcinoma (ClinicalTrials.gov: NCT02867332), have been approved but have not yet recruited patients.

PD1 knockout can also been combined with TCR modification and CAR-T cell therapy to propose a novel tumor therapeutic. For example, investigations into the effects of CRISPR-Cas9-mediated PD1- and TCR-knockout anti-mesothelin CAR-T cells on mesothelin-positive solid tumors (ClinicalTrials.gov: NCT03545815) are under way. Other clinical trials on the investigation of the responses of T cell and B cell malignancies to CRISPR-Cas9-modified CAR-T cells have been launched by the Baylor College of Medicine and Chinese People's Liberation Army (PLA) General Hospital (ClinicalTrials.gov: NCT03690011, NCT03398967, and NCT03166878). They knockout some genes on the surface of CAR-T cells to enhance anti-tumor reactivity, improve specificity to the tumor, or minimize tumor immunogenicity.

In addition, CRISPR screening identified the RIPK1 inhibitor as a potential target for amplifying anti-tumor immune reactivity. A clinical trial examining this effect on pancreatic tumors has been approved that will combine pembrolizumab with RIPK1 inhibitor

Table 1. Details of Clinical Trials Involving CRISPR-Cas9

Type of Diseases	Goal	Intervention	Start/End Date	Status	Phase	Study Type	Participants	Sponsor or/and Affiliations	ClinicalTrials.gov ID
Thalassemia	to evaluate efficiency and safety of CTX001	CTX001 (CD34+ hPSCs with CRISPR-Cas9)	Sep 2018/May 2019	recruiting	1, 2	interventional	45	CRISPR Therapeutics	NCT03655678
SCD	to evaluate efficiency and safety of CTX001	CTX001	Nov 2018/May 2022	recruiting	1, 2	interventional	45	CRISPR Therapeutics	NCT03745287
Thalassemia	to evaluate efficiency and safety of HBB-corrected iHSCs	iHSCs (HBB gene correction)	Jan 2019/Jan 2021	not yet recruiting	1	interventional	12	Allife Medical Science and Technology Co., Ltd.	NCT03728322
EBV-related malignancies	to evaluate efficiency and safety of PD1-KO CTLs	Flu, CTX, IL-2, PD1-KO EBV-CTLs	Apr 2017/Mar 2019	recruiting	1, 2	interventional	20	Baorui Liu, Nanjing Drum Tower Hospital	NCT03044743
HPV-related malignancies	to evaluate efficiency and safety of TALEN and CRISPR-Cas9	TALEN and CRISPR-Cas9	Jan 2018/Jan 2019	not yet recruiting	1	interventional	60	Hu Zheng, Sun Yat-Sen University	NCT03057912
NSCLC	to evaluate efficiency and safety of PD1-KO T cells	CTX, PD1-KO T cells	Aug 2016/Dec 2018	active, not recruiting	1	interventional	12	You Lu, Sichuan University	NCT02793856
Renal cell carcinoma	to evaluate efficiency and safety of PD1-KO T cells	IL-2, CTX, PD1-KO T cells	Nov 2016/Nov 2020	not yet recruiting	1	interventional	20	Yinglu Guo, Peking University	NCT02867332
Prostate cancer	to evaluate efficiency and safety of PD1-KO T cells	IL-2, CTX, PD1-KO T cells	Nov 2016/Dec 2020	not yet recruiting	1	interventional	20	Yinglu Guo, Peking University	NCT02867345
Bladder cancer	to evaluate efficiency and safety of PD1-KO T cells	IL-2, CTX, PD1-KO T cells	Sep 2016/Sep 2019	not yet recruiting	1	interventional	20	Yinglu Guo, Peking University	NCT02863913
Esophageal cancer	to evaluate efficiency and safety of PD1-KO T cells	PD1-KO T cells	Mar 2017/Dec 2018	recruiting	2	interventional	21	Shixiu Wu, Hangzhou Cancer Hospital	NCT03081715
Tumor of CNS	to screen and identify alleviating drugs of diseases	collection of stem cells	Nov 2015/Jun 2019	recruiting	–	observational	20	Roger Packer, Children's Research Institute	NCT03332030
Pancreatic neoplasms	to demonstrate RIPK1 inhibitor amplifies Pembro. actions	CRISPR screen, GSK3145095, Pembro.	Nov 2018/Nov 2022	not yet recruiting	2	interventional	220	GlaxoSmithKline	NCT03681951
Ovarian cancer	to develop novel tests to diagnose ovarian cancer	sample collection, CRISPR duplex sequence	Sep 2018/Sep 2019	not yet recruiting	–	interventional	25	University of Washington	NCT03606486
Mesothelin-positive solid tumors	to evaluate efficiency and safety of edited anti-mesothelin CAR-T cells	PD1- and TCR-KO/only PD1-KO anti-mesothelin CAR-T cells	Mar 2018/Jun 2019 Nov 2018/May 2020	recruiting	1	interventional	10	Han Weidong, Chinese PLA General Hospital	NCT03545815
T cell malignancies	to evaluate efficiency and safety of CD7.CAR/28zeta CAR-T cells	CD7.CAR/28zeta CAR-T cells, Flu, CTX	Mar 2019/May 2038	not yet recruiting	1	interventional	21	Rayne Rouce, Baylor College of Medicine	NCT03690011
B cell malignancies	to evaluate efficiency and safety of CD19 and CD20/CD22 CAR-T cells	CD19 and CD20 or CD22 CAR-T cells	Jan 2018/May 2022	recruiting	1, 2	interventional	80	Han Weidong, Chinese PLA General Hospital	NCT03398967

(Continued on next page)

Table 1. Continued

Type of Diseases	Goal	Intervention	Start/End Date	Status	Phase	Study Type	Participants	Sponsor or/and Affiliations	ClinicalTrials.gov ID
B cell malignancies	to monitor GVHD of allogeneic TCR- and B2M-disrupted CD19 CAR-T cells	TCR- and B2M-disrupted CD19 CAR-T cells	Jun 2017/May 2022	recruiting	1, 2	interventional	80	Han Weidong, Chinese PLA General Hospital	NCT03166878
Myeloma, melanoma, sarcoma	to evaluate efficiency and safety of CAR-T cells	NY-ESO-1 CRISPR (TCRendo and PD1) CAR-T cells, CTX, flu	Sep 2018/Jan 2033	recruiting	1	interventional	18	University of Pennsylvania	NCT03399448

EBV, Epstein-Barr virus; NSCLC, non-small cell lung cancer; KO, knockout; CTLs, cytotoxic T lymphocytes; CTX, cyclophosphamide; Flu, fludarabine; IL-2, interleukin-2; iHSCs, induced pluripotent stem cells; GVHD, graft-versus-host disease; Pembro., pembrolizumab; HPV, human papillomavirus; TCRendo, endogenous TCR.

(ClinicalTrials.gov: NCT03681951). More essential gene targets will be discovered using the CRISPR-Cas9 library that can facilitate or impair the cytotoxic effector function of immune cells and will be tested in future clinical trials. Besides modifying immune cells to treat tumors, CRISPR-Cas9 technology can be used to disrupt viral disease-causing sequences, such as human papillomavirus (HPV) 16 and HPV18 E6/E7 DNA, to prevent virus-associated cancers. A clinical trial in which CRISPR-Cas9-HPV E6/E7 plasmid gel was administrated to treat HPV persistency and HPV-related cervical intraepithelial neoplasia I has been approved (ClinicalTrials.gov: NCT03057912).

As for hereditary diseases, thalassemia and SCD are part of the CRISPR-Cas9 repertoire. Further clinical trials are investigating the efficiency and safety of CRISPR-Cas9 gene-engineered hHSCs in treating these diseases (ClinicalTrials.gov: NCT03655678, NCT03745287, and NCT03728322). What are not listed in Table 1 are the forthcoming tests of EDIT-101, a CRISPR-based experimental medicine investigated for the treatment of Leber congenital amaurosis type 10 (LCA10) by Editas Medicine, a leading genome-editing company.[70] LCA10 is a monogenic inherited retinal dystrophy disorder caused by aberrant splicing deeply due to bi-allelic loss-of-function intron 26 c.2991+1655A > G mutations in *CEP290* gene.[71] Subretinally administered EDIT-101 works to correct these splicing defects and restore wild-type *CEP290* mRNA expression through its specific CRISPR-Cas9 components packaged in AAV5 viral vector.[72] The U.S. Food and Drug Administration (FDA) has accepted the investigational new drug (IND) approval of the clinical trials of the CRISPR genome-editing therapeutic product EDIT-101, which creates a critical impetus for translation of CRISPR-Cas9 technology.[73]

These promising lines of evidence introduce an era wherein CRISPR-Cas9-mediated gene editing could be used in the clinical setting to precisely modify and control site-specific knockins without uncontrolled genomic damage.

Problems and Challenges in the Clinical Translation of CRISPR-Cas9 Technology

Despite the increasing maturity of CRISPR-Cas9 technology, its safety and efficiency are important concerns requiring comprehensive studies. Clinical translation of the CRISPR-Cas9 system is impeded by off-target alterations.[74] Specifically, gRNAs tend to have relatively high mismatch tolerance; hence, Cas9 commonly cleaves off-target sites that have sequences similar to those of target genes.[75] Efforts to increase the specificity of CRISPR-Cas9 have improved gRNA design,[76] generated new versions of the Cas9 nuclease,[77–79] and optimized delivery vehicles. Newly designed xCas9 and HypaCas9 variants are hyper-accurate without reducing target activity for precise genome editing.[79,80] Partial sequence replacement of gRNA is another strategy to prevent off-target effects without compromising on-target activity.[81] Furthermore, newly reported CRISPR-Cas inhibitors may be harnessed as effective regulators of CRISPR-Cas-mediated gene editing in the future.[82] In addition, GUIDE-seq, BLESS, HTGTS, and Digenome-seq are among the new tools designed to predict

gene-editing outcomes and identify potential off-target sites.[83,84] However, off-target alterations are only one form of genotoxicity attributable to CRISPR-Cas9 gene editing. CRISPR-Cas9 could introduce unexpected large deletions and complex rearrangements into edited cells.[85] In mouse and human cell lines, for example, large deletions amounting to 9.5 kb and complex genomic rearrangements (insertions, inversions, and combined forms) have been reported at regions adjacent and distal to targeted cut sites.[86]

Typically, CRISPR-Cas9 optimization and off-target tool design use standard human genomes as a reference. However, individual human genetic variations may lead to unexpected off-target gene editing even with well-designed gRNA.[87] Therefore, translational application of CRISPR-Cas9 must be coupled with whole-genome sequence analysis, large-scale off-target predictions, evaluation of individual intensive genotoxicity risk, and close patient monitoring.

The well-known tumor suppressor *p53* is important in CRISPR-Cas9-induced DSB repair.[88] When cells recognize CRISPR-Cas9-induced DNA damage, *p53* is activated to trigger cell-cycle arrest and kill transformed cells. Therefore, precise genome engineering in human pluripotent stem cells (hPSCs) with wild-type *p53* was less efficient than that in hPSCs with inhibited *p53*.[89] These findings suggest that successfully transformed cells would be mostly *p53* inhibited and may exhibit unknown characteristics that could even increase tumorigenesis potency. Thus, more research is necessary to understand how CRISPR-Cas9 technology affects *p53*.

The immunogenicity of Cas9 nuclease is another concern that should be considered during clinical translation of CRISPR-Cas9 technology. The serum of some donors naturally contains Cas9 antibodies, with 79% exhibiting anti-saCas9 and 65% exhibiting anti-SpCas9.[90] 96% of the donors evaluated (46/48 in the study) showed a preexisting T cell immune memory against SpCas9.[91] Human anti-Cas9 immune response causes low editing efficiency and could lead to a serious immune storm in patients receiving CRISPR-Cas9 treatment.

A final serious concern is the generalizability and efficiency of CRISPR-Cas9 technology. For instance, protospacer adjacent modif (PAM; NGG) sequences are necessary at target sites for accurate CRISPR-Cas9 genome editing. Until recently, existing Cas9 has been recognized in only a few PAM sequences, limiting extensive application of this technology. Fortunately, newly developed xCas9 can recognize a broader range of PAM sequences (NG, GAA, and GAT), quadrupling the scope for the application of CRISPR-Cas9 technology.[79] In addition, precise CRISPR-Cas9-mediated genomic insertions experience low efficiency via HDR;[29] however, this efficiency can be enhanced through suppression of NHEJ, nucleofection delivery and the use of a single-stranded DNA (ssDNA) donor instead of double-stranded DNA (dsDNA).[68,92,93]

In conclusion, further studies are required to clarify the safety and efficiency of CRISPR-Cas9 before the technology is adaptive to clinical settings.

Conclusions and Future Directions

The rapidly developing suite of CRISPR-Cas9-mediated gene-editing technologies is yielding enormously beneficial results in the modern life sciences. These tools allow for precise genomic modifications, transcriptional regulation, and epigenetic editing with high efficiency, generality, and simplicity. Undoubtedly, CRISPR systems are endowed with the potential to revolutionize the fields of genetics and medicine. The past few years have witnessed the introduction of metadata era with large-scale analyses of genome sequences,[94,95] deep sequencing technologies,[96,97] and single-cell transcriptomics.[98] CRISPR-based technologies are suggested to play a major role in this metadata revolution. The technologies will help in understanding specific gene functions through their ability to precisely dissect genetic networks. Knowledge thus obtained can then be leveraged for personalized treatment of human diseases. Furthermore, CRISPR-Cas9 has many potential therapeutic applications. In 2017 and 2018, CRISPR-based therapeutic applications were assessed in large animals and human embryos, and more CRISPR-related clinical trials have been launched since then. However, numerous existing controversies, e.g., potential off-target effects, the carcinogenic effect of CRISPR components, and the immunogenicity of Cas9 nucleases, warrant comprehensive scientific explanations. Although efforts have been focused on optimizing the technologies and answering these questions, all these answers have become vague because of the emergence of new questions. Therefore, future studies are required to focus on in-depth understanding of intracellular mechanisms underlying CRISPR-Cas9-mediated gene editing for appropriate clinical application of such technologies. Besides this, CRISPR-related clinical trials should be responsibly supervised and controlled.

In addition, social and ethical concerns regarding the application of CRISPR-Cas9 technology deserve public consideration. The first CRISPR gene-engineered twins worldwide, born in China, have caused an ethical uproar. Whether human genomes can be altered in somatic cells or embryos to suit their requirements is a controversial issue. More problems will arise if CRISPR-Cas9 gene-edited babies are born. For example, undetectable off-target effects that have not yet been studied comprehensively would change human genomes thoroughly generation after generation and could even cause disastrous consequences. Worries also include those related to the mental health and social behavior of the babies. as well as the designed human capabilities, such as intelligence, appearance, and personality. More worryingly, utilization of such technologies by profitable individuals would cause more profound and broader social issues. In the future, the developing of CRISPR-Cas9 technology must be accompanied by organized and rigid supervisory policies to minimize the preceding problems.

However, translational research of CRISPR-Cas9 technology in biomedicine is imperative and should first target advanced tumors and severe hereditary diseases, despite the preceding ethical concerns. As a versatile and convenient nucleic acid manipulation platform, the CRISPR-Cas9 system can be harnessed to study molecular mechanisms in the process of disease genesis and progression and, more

Table 2. Detailed Information about the Representative Examples Summarized in the Text

Sequecnce No	Target Gene	gRNA Sequence	Delivery Methods	Efficiency	Off-target	References
1	Dmd (mouse)	*L8: ATAATTTCTATTATATTACA; R3: ATTTCAGGTAAGCCGAGGTT*	viral delivery (IM)	~80%	No detectable off-targets (top 10)	35
2	HBB (hPSCs)	GACCCAGAGGTTGAGTCCTT	electroporation nucleofection	75%	No detectable off-targets (top 10)	41
3	MYBPC3 (embryos)	GGGTGGAGTTTGTGAAGTAT	electroporation nucleofection (iPSCs) oocytes/zygote microinjection (embryos)	72.2%	No detectable off-targets	49
4	Pcsk9 or Hpd (*in utero*)	Pcsk9: CAGGTTCCATGGGATGCTCT	viral delivery (fetal injection)	10 to ~15%	Pcsk9: no detectable off-targets (top 9)	50
		Hpd: CATTCAACGTCACAACCACC		~15%	Hpd: no detectable off-targets (top 10)	
5	SOD1	1: GCCTGCATGGATTCCATGTTCATGAGT	viral delivery (facial vein injection)	NS	No significant off-targets	52
		2: GGCCTGCATGGATTCCATGTTCATGAGT				
6	PD1 (human primary T cell)	1: GGCGTGACTTCCACATGAGCGTGG	electroporation nucleofection	1: 61.9%; 2: 52.6%	NS	68
		2: GGCAGTTGTGTGACACGGAAGCGG		3: 40%		
		3: GACAGCGGCACCTACCTCTGTGG		4: 52.6%		
		4: GGGCCCTGACCACGCTCATGTGG				
7	TCR (human primary T cell)	1: ACCCGAGGTCGCTGTGTTTG	viral delivery	>90% (four gRNAs in tested cell line), sg1 was selected	NS	59
		2: AGGTCGCTGTGTPTGAGCCA				
		3: CGACCACGTGGAGCTGAGCT				
		4: GATACTGCCTGAGCAGCCGC				
8	CD33 (hPSCs)	4: GAGTCAGTGACGGTACAGGA	viral delivery	65%	No significant off-targets	60
9	TRAC, TRBC, B2M, PD1	TRAC-1: AGAGTCTCTCAGCTGGTACA	electroporation nucleofection	TRAC: 81.7%; TRBC: 49.3%	No significant off-targets	63
		TRAC-2: TGTGCTAGACATGAGGTCTA		B2M: 79.9%		
		TRBC-1: GCAGTATCTGGAGTCATTGA		PD1: NS		
		TRBC-2: GGAGAATGACGAGTGGACCC				
		B2M: CGCGAGCACAGCTAAGGCCA				
		PD1: GGCCAGGATGGTTCTTAGGT				

B2M, Beta-2-Microglobulin; IM, intramuscular injection; IV, intravenous injection; iPSCs, induced pluripotent stem cells; TRBC, T cell receptor β constant.

significantly, to remedy or correct disease-causing gene mutations for clinical translational applications in major human diseases, especially cancers. Present preclinical explorations and future directions of translational applications primarily include these three aspects: (1) genetically engineer the tumor genome to inhibit tumor cell growth and proliferation and induce more necrosis and apoptosis, or to enhance tumor cells' sensitivity to radiotherapy and some chemotherapeutics; (2) genetically engineer immune cells' genome to increase the secretion of toxic cytokines, or to rescue immune cells from functional inhibition; and (2) genetically engineer normal cells, e.g., bone marrow cells, to protect them from the toxic effects of radiotherapy and chemotherapy. As has been illustrated in this review, recent years have witnessed deepening functional dissection and the ever-expanding translation prospect of CRISPR-Cas9 tools, yet translating these into the clinic remains intractable. Safety is one of those factors that hold us back, including uncontrolled off-target effects, potential immunogenicity, and immunological adverse effects. Efficiency is another one, because advanced-tumor patients after multiline therapy have insufficient immunity and anergic even exhausted immune cells.

Altogether, notwithstanding significant advancements and newly emerging challenges for CRISPR-Cas9 technology, major improvements and therapeutic applications can be envisioned.

Data Resources
Detailed information about the examples summarized in the text is listed in Table 2 and the ClinicalTrials.gov database: https://clinicaltrials.gov/.

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