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Review

Disease modeling and stem cell immunoengineering in regenerative medicine using CRISPR/Cas9 systems

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ABSTRACT

CRISPR/Cas systems are popular genome editing tools that belong to a class of programmable nucleases and have enabled tremendous progress in the field of regenerative medicine. We here outline the structural and molecular frameworks of the well-characterized type II CRISPR system and several computational tools intended to facilitate experimental designs. The use of CRISPR tools to generate disease models has advanced research into the molecular aspects of disease conditions, including unraveling the molecular basis of immune rejection. Advances in regenerative medicine have been hindered by major histocompatibility complex-human leukocyte antigen (HLA) genes, which pose a major barrier to cell- or tissue-based transplantation. Based on progress in CRISPR, including in recent clinical trials, we hypothesize that the generation of universal donor immune-engineered stem cells is now a realistic approach to tackling a multitude of disease conditions.

Contents

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1. Introduction

Currently trending genome-editing tools support the study of gene functions and disease modeling by altering genes with programmable nucleases to mimic uncharacterized genotypes. The ability to genetically manipulate biological systems holds enormous potential across biotechnology, basic science, and medicine. The CRISPR/Cas9 system belongs to a class of programmable nucleases and permits precise alteration of eukaryotic genomes. These nucleases act by inducing targeted double-stranded DNA breaks (DSBs) on chromosomes, followed by activation of the homology-directed repair (HDR) pathway. The HDR pathway requires exogenous DNA with short sequences homologous to the acceptor sites to act as templates for repairing the underlying sequence-specific DSBs. Activation of the endogenous HDR pathway can precisely alter the genome at predefined sequences [1,2]. However, despite its enormous potential, use of HDR-gene editing approaches is limited by the predominance of the non-homologous end joining (NHEJ) pathway in repair of DSBs. The NHEJ pathway has been used to study gene functions via disruption of expression due to formation of indels (insertions or deletions). However, CRISPR systems have been reported to utilize this pathway to restore the functions of genes disrupted by disease mutations. For example, CRISPR systems have been used to repair *CYBB* gene mutations causing X-linked chronic granulomatous disease (XCGD) [3] and *FANCA* mutations causing Fanconi anemia (FA) [4]. The HDR pathway is restricted mainly to the G2-S phases of the cell cycle and depends on availability of sister chromatids, whereas the NHEJ pathway involves end-to-end ligation of DSBs and takes place throughout the cell cycle [5,6]. Reversible cell synchronization in the G2/M phase has been shown to improve donor integration efficiency during gene editing [7]. Inhibition of the ligase IV-dependent NHEJ DNA repair pathway by small molecule inhibitors like SCR7 has demonstrated an increase in homologous recombination (HR) in mammalian cell lines and mouse models [8–10]. Therefore, control of delivery time of the CRISPR/Cas9 system along with inhibition of the NHEJ repair mechanism can greatly improve genome editing efficiency. Various small molecules that can enhance HDR efficiency are listed in Table 1.

In this review, we outline the structural and molecular frameworks of the well-characterized type II CRISPR system and several computational tools intended to facilitate experimental design. The gene-editing approaches have been expanded to progenitor cells, including induced pluripotent stem cells, and *in vivo* in a wide array of higher mammals developed as disease models, which we discuss in this review.

Table 1
Summary of HDR-enhancing small molecules.

Small Molecule	Target	Mechanism	Effect on HDR	Reference
SCR7	Inhibition of NHEJ	Inhibition of ligase IV	Decrease NHEJ repair and increase HDR in several cell lines and mice and rats with ds and ss donors	[9–14]
i53	Inhibition of NHEJ	Inhibition of 53BP1, an important regulator of the DSB repair pathway	Increased HDR by 5–6 fold with ss and ds donors	[15]
STL127705	Inhibition of NHEJ	Inhibitor of the DNA repair protein Ku70/80	2.8 to 7.2-fold and 2.3 to 4-fold increase in genome editing with Cas9n and Cpf1, respectively	[16,17]
KU0060648	Inhibition of NHEJ	Inhibition of DNA-protein kinase catalytic subunits	Increased HDR in HEK293T cells with reduced NHEJ frequency	[18]
NU7026	Inhibition of NHEJ	Inhibition of DNA-protein kinase catalytic subunits	Reduced NHEJ frequency with increased KI efficiency in hiPSCs using Cas9 or Cpf1	[19]
NU7441	Inhibition of NHEJ	Inhibition of DNA-protein kinase catalytic subunits	Increased HDR in HEK293T and hiPSCs by 13.4 fold in zebrafish embryos	[18,20,21]
VE-822	Inhibition of NHEJ	Inhibition of ataxia telangiectasia mutated and Rad3-related kinase (ATR)	5.9-fold increased HDR in hiPSCs with ss and ds donors after DSB induced by Cpf1	[22]
AZD7762	Inhibition of NHEJ	Inhibitor of checkpoint kinase CHEK1	2.7-fold increased HDR in hiPSCs with ss and ds donors after DSB induced by Cpf1	[22]
M3814	Inhibition of NHEJ	Inhibition of DNA-protein kinase catalytic subunits	Increased KI in hiPSC and K562 cells using Cas9 or Cpf1	[23]
RS-1	Direct increase of HDR	Enhance binding of RAD51 with ssDNA	Increased HDR in HEK293T cells, HeLa cells, and rabbit and bovine embryos	[24–27]
Mimosin, thymidine, hydroxy urea, lovastatin	Regulation of cell cycle	G1/S blocker	Increased HDR in neonatal fibroblasts	[6]
Nocodazole	Regulation of cell cycle	G2/M blocker	Increased HDR in HEK293T and hPSC using ss or ds donors	[6,20]
XL413	Regulation of cell cycle	G1/S blocker	Increased HDR in HSPCs, K562 cells, and T cells with ss or ds donors	[28]
Aphidicolin	Regulation of cell cycle	G1/S blocker	Increased HDR in HEK293T and neonatal fibroblasts using ss donor	[6]
ABT263	Regulation of cell survival	BCL inhibitor	70% increase in HDR in hiPSC	[29]
ABT751	Regulation of cell cycle	G2/M blocker	Increased HDR in hPSCs with ds donor	[7]
Valproic acid (VPA)	Undetermined pathway	Histone deacetylase inhibitor	Increased HDR in hESCs with ds donor	[30]
Resveratrol	Suppression of NHEJ	Downregulation of LIG4, PRKDC, KU70, and KU80	Increased HDR in porcine fetal fibroblast using ds donor	[14]
Brefeldin A	Undetermined pathway	Inhibition of intracellular transport from ER to golgi	Increased HDR in mouse ESCs using ds donor	[31]
L755507	Undermined pathway	Agonist for β3-adrenergic receptor	Increased HDR in mouse ESCs using ds donor	[14,31]

**NHEJ – non-homologous end joining; HDR – homology-directed repair; ss – single stranded; ds – double stranded; hiPSCs – human-induced pluripotent stem cells; hPSCs – human pluripotent stem cells; ESCs – embryonic stem cell; KI – knock-in; ER – endoplasmic reticulum.

2. Programmable nucleases

DSB-inducing programmable nucleases facilitate precise editing at endogenous genomic loci to enable methodical interrogation of genes and gene variations in a broad range of species [32]. Programmable nucleases include the first truly targetable zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the popular RNA-guided engineered nucleases (RGENs), the most famous of which is the bacterial and archaean clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated system (Cas) [1,33–36]. Though these nucleases have unique characteristics, they share a common mode of action: cleaving chromosomal DNA at specific sites to trigger endogenous DNA repair mechanisms and produce targeted genome modifications. In this section, we review the development of and advances in the popular CRISPR/Cas endonucleases.

2.1. CRISPR system: A new generation of programmable nucleases

CRISPR/Cas systems have emerged as an efficient, potentially facile alternative to ZFNs and TALENs for inducing DSB-mediated gene alterations [37]. The CRISPR system is a part of the bacterial and archaean acquired immune system that identifies and destroys foreign DNA via RNA-guided DNA cleavage [38]. These systems have been classified into three types (I-III) across various bacterial and archaeal hosts. CRISPR systems comprise Cas genes; non-coding RNAs; and distinctive, repetitive elements (direct repeats) interspersed with short variable sequences called *protospacers* that together constitute the CRISPR RNA (crRNA) [38,39]. Each protospacer is associated with a protospacer adjacent motif (PAM) that is responsible for sequence identification and varies depending on CRISPR system [40,41]. CRISPR/Cas systems assemble mature crRNA with Cas proteins to form crRNA-effector complexes that interrogate target DNA and destroy matching sequences in foreign genomic material [42]. By manipulating the guide RNA (gRNA) component of the crRNA, a CRISPR/Cas system can be directed to target virtually any DNA sequence, and it has been shown to be applicable to human cells [43,44].

The best characterized CRISPR systems are the type II systems, which include the Cas9 nucleases, gRNA encoding the crRNA array, and *trans*-activating crRNA (tracrRNA). TracrRNA is required to process the crRNA assembly into discrete units containing the 20 bp guide sequences required for Cas9 specificity and partial direct repeats [36,45]. *Streptococcus pyogenes*-derived CRISPR/Cas (spCas/SpyCas9) targets DNA that contains 5′-NGG-3′ as its PAM. On the other hand, CRISPR/Cas derived from *S. thermophilus* targets 5′-NNAGAA-3′ for CRISPR1 (st1Cas9) and 5′-NGGNG-3′ (st3Cas9) for CRISPR3; *Staphylococcus aureus* (SaCas9) targets 5′-NNGRT-3′; *Acidaminococcus* sp. (Cas12a) and *Lachnospiraceae bacterium* (Cpf1) recognize 5′-TTTN-3′, and *Neisseria meningitidis* recognizes 5′-NNNNGATT-3′ [47–53].

2.1.1. Molecular structure of the CRISPR/Cas9 system

The popularity of the CRISPR/spCas9 system has been attributed to its simplicity in designing single guide RNAs (sgRNAs) via synthetic fusion of tracrRNA and crRNA, which can be modified to cleave virtually any sequence preceding the 5′-NGG-3′ PAM sequence [50,51]. These RNA-guided nuclease functions can be performed in mammalian cells via heterologous expression of the requisite human codon-optimized RNA components and optimized Cas9 [54]. Analyses of the Cas9 crystal structure have revealed two lobes, a recognition (REC) lobe and a nuclease (NUC) lobe, with adjacent active sites. The surface area between the two structural lobes was reported to be 1034 Å [55]. A schematic representation of the CRISPR/Cas9 system is shown in Fig. 1A.

The REC lobe consists of a long bridge helix (residues 60–93), REC1 domain (residues 94–179 and 308–718), and REC2 domain (residues 180–307). The REC1 protein consists of 25 α helices (α2-α5 and α12-α32) and two β sheets (β6, β10 and β7-β9) and adopts an elongated α-helical structure, whereas REC2 adopts a six-helix bundle (α6-α11) structure. On the other hand, the NUC lobe contains a structural core that forms the RuvC domain (residues 1–59, 718–769, and 909–1098), HNH domain (residues 775–908), and the PAM-interacting (PI) domain (residues 1099–1368) (Fig. 1B) [56]. The RuvC domain consists of three RuvC motifs (RuvC I-III) made up of six mixed β sheets flanked by α helices; it has a positive charge due to its interface with the PI domain, and it interacts with the sgRNA tail. A structural comparison analysis identified four catalytic residues (aspartic acid 10 (Asp10), glutamic acid 762 (Glu762), histidine 983 (His983), and Asp986) in the Cas9 RuvC domain that are structurally similar to an RNase H fold found in members of the retroviral integrase superfamily, suggesting a two-metal-ion catalytic cleavage mechanism for non-target DNA cleavage [55,56]. Asp10 is reported to be critical for non-complementary DNA cleavage, and Cas9 cleavage activity depends on Mg^{2+} ions [56–58]. The HNH domain has only a few contact points with the rest of the protein, lies between the RuvC II and III motifs, and comprises four α helices that surround dual-stranded antiparallel β sheets [56]. The HNH nuclease active site is made up of three catalytic residues (eg-Asp40, His41, and Asn62) and is said to cleave DNA via a single metal mechanism [59]. The crystal structure of *Streptococcus pyogenes* CRISPR/Cas9 endonuclease in complex with sgRNA is shown in Fig. 1C. The REC and NUC lobes are connected by two link segments, one of which forms an arginine-rich bridge. Because sgRNA and its target DNA heteroduplex are negatively charged, they interact in the positively charged groove between the REC and NUC lobes. Whereas the REC-recognition lobe is essential for binding sgRNA and DNA, the NUC-nuclease lobe contains the HNH and RuvC nucleases that cleave the complementary and non-complimentary strands of the target DNA, respectively. Alignment of the bound complementary DNA and the RuvC domain active site suggests that the PI domain recognizes PAM sequences in non-complementary DNA (Fig. 1A and 1C) [56].

Cas9 has also been reported to be mutable at either HNH (H840A) or RuvC (D10A) and transforms it into a nickase capable of single-strand nicks. However, mutating both nuclease domains yields dead Cas9 (dCas9) that retains its RNA-guided DNA binding ability but has no cleavage activity [60].

2.1.2. Mechanism of action of CRISPR/Cas9

Cas9 is a powerful, highly specific, and efficient genome editing tool that has been studied extensively. Structural and mechanistic studies have provided a fundamental understanding of the mechanism that regulates the CRISPR/Cas9 system, along with a framework for structure-based rational design to improve its efficiency and minimize its off-target results.

Formation of the Cas9-sgRNA complex is important for the transition from inactive to active conformation of Cas9 upon PAM recognition [36,50]. Accurate sgRNA-target DNA complementarity is necessary for Cas9-mediated targeting and cleavage of DNA, though imperfect base pairing at non-seed regions is tolerated within target binding specificity [61]. Cas9 forms a Cas9-sgRNA binary complex by recognizing the PAM-proximal guide region and repeat: anti-repeat sgRNA duplex. This Cas9-sgRNA complex searches DNA sites for complementary target sequences and requires complementary base pairing between the 20-bp spacer sequence and the protospacer in the target DNA and the presence of a PAM sequence adjacent to the target site to form the final Cas9-sgRNA-target DNA ternary complex [62]. Interaction between the Cas9 complex and the target DNA occurs through a three-

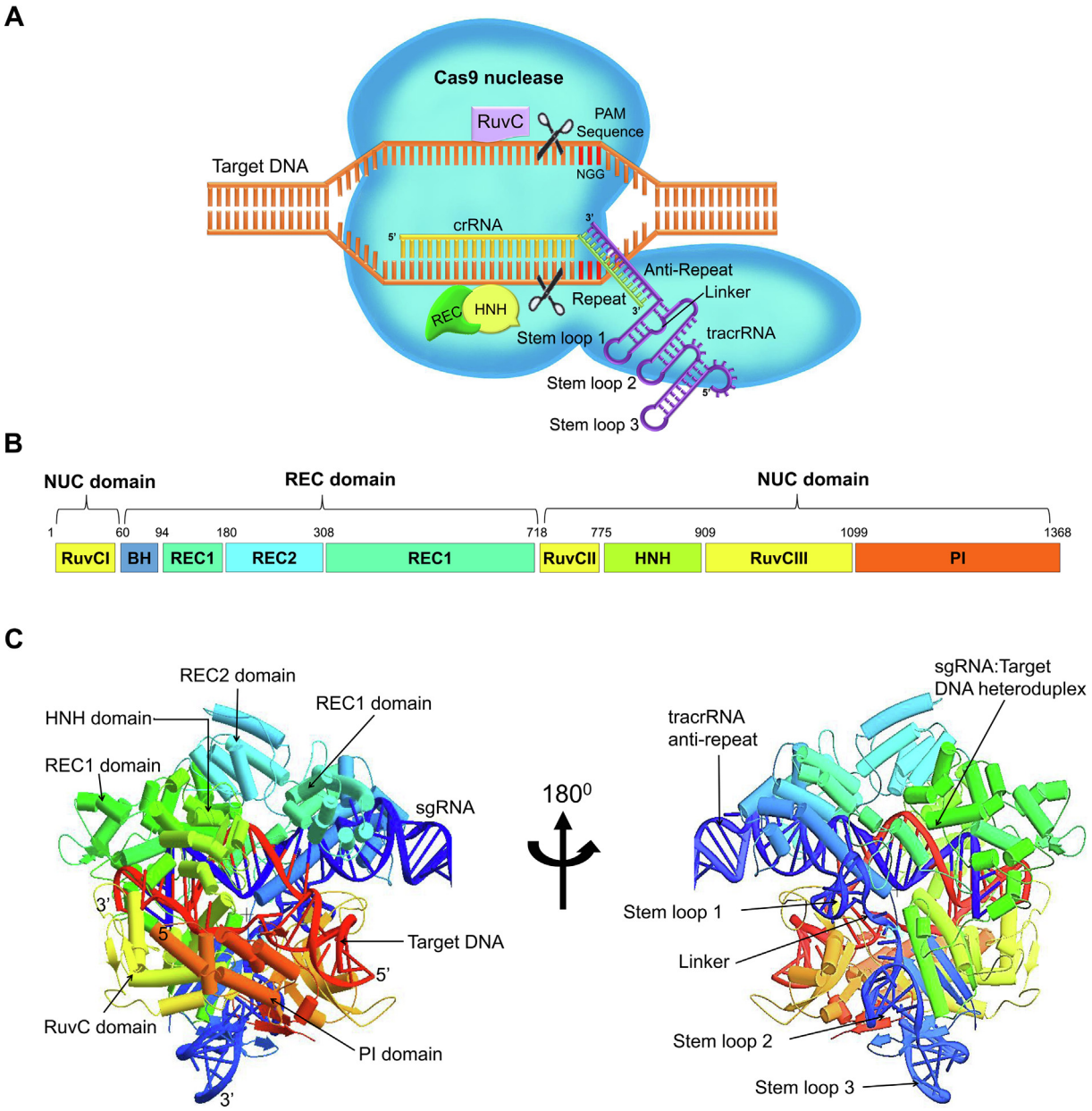

Fig. 1. Structure of *Streptococcus pyogenes* Cas9-sgRNA complex. A. Schematic representation of the CRISPR/Cas9 system. B. Domain organization of the spCas system. The bilobed architecture of Cas9 consists of a recognition (REC) domain and a nuclease (NUC) lobe. C. Crystal structure of spCRISPR/Cas9 endonuclease in complex with sgRNA and double-stranded target DNA primed for specific DNA cleavage (PDB id: 5F9R). The REC domain consists of three regions, a long α helix called bridge helix (BH) and the REC1 and REC2 domains, while the NUC domain consists of RuvC, HNH, and PAM-interacting (PI) domains. The REC1 and REC2 domains interact with repeat-anti-repeat duplex and are essential for recognition of sgRNA and target DNA. The PI domain recognizes a PAM sequence on a non-complementary DNA strand. The RuvC domain is assembled into three split motifs containing an RNase H fold and interfaces with the PI domain to form a positively charged surface that interacts with the 3′ tail of sgRNA. The HNH and RuvC nuclease domains are essential for cleavage specificity of complementary and non-complementary strands of the target DNA, respectively [56].

dimensional collision that allows rapid dissociation of Cas9 from DNA that does not contain the right PAM sequences. This dwell time depends on gRNA complementarity with the adjacent DNA when the correct PAM is present [63–65].

The PI domain recognizes PAM sequences on non-complementary DNA to form the 20-bp guide-target DNA heteroduplex formation recognized by Cas9 in a sequence-independent manner and triggers local DNA melting at PAM-adjacent nucleation sites prior to formation of the ternary complex. Next, RNA strand invasion forms an RNA-DNA hybrid and displaces a DNA R-loop strand from the PAM-proximal end to the PAM-distal end [65,66]. The REC1 (Asn497, Trp659, Arg661, and Gln695), RuvC (Gln926), and PI (Glu1108) domains interact with the phosphate backbone groups of the target DNA (nucleotides 1′, 9′-11′, 13′, and 20′). The REC1 (Leu169, Tyr450, Met495, Met694, and His698) and RuvC (Ala728) domains interact via Van der Waals interactions with the C2′ atoms of the target DNA at nucleotides 5′, 7′, 8′, 11′, 19′, and 20′. Assembly of the ternary complex follows cleavage of the complementary strand by the HNH domain and cleavage of the non-complementary strand by the RuvC domain in the sgRNA-target heteroduplex complex [56,61,67]. Each domain cleaves a strand of the DNA three base pairs from the NGG PAM, producing a blunt-ended DSB [65].

Post cleavage, Cas9 remains bound to the target DNA until it is displaced by other cellular processes for recycling [65,68]. Cas9 nickase produces single-strand breaks by targeting only a single

Table 2
Available tools to design sgRNA and to evaluate on-target efficiency/off-target effect.

Tool name	Description	On-target prediction	Off-target prediction	Source	Reference
AttnToCrispr	To determine CRISPR Cas9 and Cas12a specificity and efficiency	Yes	Yes	https://github.com/qiaoliuhub/AttnToCrispr	[79]
Breaking-Cas	To design sgRNA for SpCas9, SaCas9, and Cpf1 and to evaluate probable off target sites	Yes	Yes	https://bioinfogp.cnb.csic.es/	[80]
CHOPCHOP	Selection of the optimal CRISPR/cas9 sequence gene; identifying sgRNA targeting Cas9 and its variants and Cpf1.	Yes	Yes	https://chopchop.cbu.uib.no	[81–83]
CRISPR-RGEN Tools	Potential off-target sites in input sequence or given genome	No	Yes	http://www.rgenome.net/	[84–86]
CRISPOR	To identify sgRNA sequence in input, rank them according to efficiency score, and predict off-target and on-target activity	Yes	Yes	http://crispor.tefor.net/	[87,88]
CCTop	Identifying sgRNA targeting Cas9 and its variants and Cpf1.	Yes	Yes	https://crispr.cos.uni-heidelberg.de/	[89]
CRISPR-ERA	To design sgRNAs for genome editing, repression, and activation	Yes	No	http://crispr-era.stanford.edu/	[90]
CRISPRdirect	To design sgRNAs	No	Yes	https://crispr.dbcls.jp/	[91]
CRISPRscan	To design sgRNAs for Cas9 and Cpf1	Yes	Yes	https://www.crisprscan.org/	[92]
CRISPETa	Flexible and scalable paired sgRNA design based on an empirical scoring model	Yes	Yes	http://crispeta.crg.eu/	[93]
CRISPRseek	To design sgRNAs for target sequences identified in wide variety of genome-wide analyses	Yes	Yes	http://bioconductor.org/	[94]
CRISPR-GE	Experimental design and mutation analysis for genome editing in the Cpf1/CRISPR/Cas9 system	Yes	Yes	http://skl.scau.edu.cn/	[95]
Cas-OFFFinder	To identify potential off-target sites in a given genome or user-defined sequences	No	Yes	http://www.rgenome.net/cas-offinder/	[86]
CLD	To predict a large fraction of functional sgRNAs	Yes	Yes	https://github.com/boutroslab/cld	[96]
CFD	Off-target evaluation	No	Yes	https://portals.broadinstitute.org/gpp/public/software/index	[97]
CRISTA	To design sgRNAs for targeting sequences identified in a wide variety of genome-wide analyses	Yes	Yes	http://crista.tau.ac.il/	[98]
CRISPR-OFF	Off-target evaluation	No	Yes	https://rth.dk/resources/crispr/	[99]
CRISPR optimal target finder	Off-target evaluation	No	Yes	http://targetfinder.flycrispr.neuro.brown.edu/	[100]
COSMID	To identify potential off-target sites with the specified number of mismatched bases and insertions or deletions compared with the guide strand	Yes	Yes	https://crispr.bme.gatech.edu/	[101]
CRISPR.mit	To design sgRNAs and evaluate off-target effects	Yes	Yes	https://zlab.bio/guide-design-resources	[60]
CROP-IT	To design sgRNAs and evaluate off-target effects	Yes	Yes	http://cheetah.bioch.virginia.edu/AdliLab/CROP-IT/homepage.html	[102]
CRISPR MultiTargeter	To identify unique isoform-specific sgRNA sites and common sgRNA sites	Yes	Yes	http://www.multicrispr.net/	[103]
CASPER	To accommodate use of the CRISPR/Cas system for genome editing, multitargeting analysis, and multispecies population analysis.	Yes	Yes	https://github.com/TrinhLab/CASPER	[104]
CRISPy-web	To design sgRNAs for a user-provided microbial genome (non-model organisms)	Yes	Yes	https://crispy.secondarymetabolites.org/	[105]
CRISPR-PLANT	To evaluate off-target sites for seven genomes of model and crop plants	Yes	Yes	https://www.genome.arizona.edu/crispr2/	[106,107]
DeepCRISPR	To design sgRNAs with on-target and off-target site prediction with deep learning	Yes	Yes	http://www.deepcrispr.net/	[108]
E-CRISP	To design sgRNAs	Yes	Yes	http://www.e-crisp.org/E-CRISP/	[109]
EuPaGDT	To design gRNAs for eukaryotic pathogens	Yes	Yes	http://grna.ctegd.uga.edu	[110]
Elevation	Cloud-based machine learning platform for off-target and on-target prediction	Yes	Yes	https://crispr.ml/	[111]
FlashFry	To design sgRNAs and predict on-target and off-target sites with unconstrained number of mismatches	Yes	Yes	http://mckennalab.org/FlashFry/	[112]
GuideScan	To design high-density sets of sgRNAs for single and paired sgRNA genome-wide screens and correctly determine off-target sites	Yes	Yes	http://www.guidescan.com/	[113]
GT-Scan	To identify unique genome targets			https://gt-scan.csiro.au/	[114]
PROTOSPACER	Offline software for flexible design of Cas9 sgRNAs with a graphical user interface with a file-based database and third-party sequence mapping tools to maximize flexibility and information retrieval when designing sgRNAs	Yes	Yes	http://www.protospacer.com/	[115]
SgRNA designer	To design sgRNAs for SaCas9 and SpCas9	Yes	Yes	https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design	[97,116]
sgRNAcas9	Design of gRNAs for Cas9s from *S. aureus* and *S. thermophilus* 3 and Cpf1	Yes	Yes	http://www.biootools.com/software.html	[117]
sgRNA Scorer 2.0	Design of gRNAs for Cas9s from *S. aureus* and *S. thermophilus* 3 and Cpf1	Yes	No	http://crispr.med.harvard.edu/sgRNAScorerV2	[118]
WU-CRISPR	To design high-density sets of sgRNAs for single and paired sgRNA genome-wide screens and correctly determine off-target sites	Yes	Yes	http://crispr.wustl.edu	[97]

strand of the DNA duplex. Staggered-end DNA can be generated by pairing sense and anti-sense targeting sgRNAs with Cas9 nickases. DSBs generated by such double nicks can enhance the specificity of genome editing [68]. DNA cleavage can be repaired by either the NHEJ pathway or the HDR pathway.

2.1.3. Tools to design sgRNAs

sgRNA design is critical to CRISPR-based screening techniques. Each sgRNA contains a target DNA complementary spacer sequence that guides the Cas9 protein to the target sequences. The cleavage efficiency and binding of the CRISPR system require different complementarity status within the 20 bp region. sgRNA design aims to identify specific target sites in the genome by simply scanning PAM sequences, such as the 5′-NGG-3′ of spCas9, but that process faces several challenges. Currently, many tools have been developed for sgRNA design, and they vary in design specifications, genomes, parameters, and so on [69,70]. Complementarity between the 5′-end 20-nt sgRNA sequence and the target DNA should theoretically be sufficient for sgRNA-Cas9 complex formation and cleavage, but previous studies suggest that sgRNAs have variable cleavage efficiencies. Researchers need to select the best sgRNAs as input sequences by identifying potential off targets and their relative cleavage rates, and that process can be facilitated by the many computational tools summarized in Table 2. Highly stable binding requires seven to nine matched bases proximal to the PAM region; even a few mismatches in this region can hinder cleavage. As few as four mismatches of up to eleven bps in length in the PAM-proximal region have been shown to hinder cleavage but not binding by allowing formation of stable complexes. This suggests an extremely slow rejection that could sequester Cas9-RNA [71,72]. The off-target effects of sgRNA have been widely investigated using *in vitro* genome-wide assays that align spacer sequences to the genome [60] with LAM-HTGTS [73], Digenome-seq [74], SITE-Seq [75], CIRCLE-seq [76], DISCOVER-seq [77], dCas9 ChIP-seq [61], and GUIDE-seq [78]. Although the studies reached different conclusions based on analysis method, they all agreed on the importance of PAM, the 7–10-nucleotide seed sequence proximal to PAM, and the concentrations of Cas9 and gRNA for efficient cleavage. Tools designed to aid sgRNA designing by analyzing their efficiencies are summarized in Table 2.

To further improve DNA targeting efficiency by processing only correct targets, systematic investigations into the binding kinetics of Cas9-RNA need to be conducted. How dissociation kinetics vary according to mismatches also needs to be established to determine the rejection rates of mismatched/partially matched sequences [71].

One target efficiency detection model was proposed by Doench et al., who created a pool of sgRNAs and screened all possible target sites across six endogenous mouse and three endogenous human genes. They used antibody staining and flow cytometry to quantitatively assess 1841 sgRNAs for ability to produce null target gene alleles. Using those data, they generated a predictive model of sgRNA activity to improve sgRNA design for gene screening and editing. That model was made into an online tool that rates active sgRNAs targeting any gene of interest with score between 0 and 1, with higher values indicating higher efficiency [119]. Several tools (CHOPCHOP, E-CRISP, WU-CRISPR, CRISPR library designer, CRISPRpred, CRISPETa, and CRISPOR [81,83,88,93,96,109,120,121]) use that model, which is driven by the hypothesis that sgRNA knockout efficiency can be empirically scored considering the effects of genome context factors. Another sgRNA design method predicts sgRNA efficiencies considering features from a training model. Available tools using that method include SSC, CRISPRscan, sgRNA Designer, and Deep CRISPR [92,97,108,118,122,123]. These comprehensive computational platforms unify sgRNA on- and off-target predictive scores into one framework with deep learning and surpass performance of state-of-the-art *in silico* tools.

The advances in CRISPR techniques and volume of genome-editing data being accumulated are presenting new computational challenges. The *in silico* design of sgRNAs has become a key issue for successful gene-editing using CRISPR systems, and continuous efforts are being made to refine it [124]. sgRNA efficiency is significantly influenced by the nucleotide composition of DNA proximal to or downstream of PAM. Nucleotides upstream of PAM do not significantly affect sgRNA efficiency. For efficient sgRNA design associated with Cas9, human and mouse cell lines prefer guanines at the −1 and −2 positions proximal to PAM. On the other hand, thymidine at the + 4/-4 positions is disfavored close to PAM [122,125,126]. Efficient *in silico* models that integrate heterogeneous genome editing data can be built to derive unbiased sgRNA design rules and improve sgRNA design [119]. A recent development in sgRNA design involves an *in vivo* library-on-library method that can simultaneously assess sgRNA activity across about 1400 genomic loci to unravel underlying epigenetic parameters and nucleotide sequences related to sgRNA knockout (KO). Such research has found that sequence composition and locus accessibility are important factors of sgRNA activity [118].

Given the number of features involved, data modeling most commonly uses machine learning methods. The construction of such models requires that many sgRNAs be tested experimentally to build a robust dataset that can enable prediction of efficiency. Therefore, researchers have typically adopted biological enrichment schemes that monitor events affecting observable biological phenotypes such as cell survival [127]. Other factors that influence sgRNA activity can be discovered by incorporating additional datasets, modeling approaches, and activity readouts. All those features have been shown to correlate with the on-target activity of Cas9-sgRNA complexes, so they can help enable effective use of CRISPR technology to probe gene functions and edit genomes.

2.2. Recent development in CRISPR/Cas system for improved genome editing

Based on recent advances in crystal structure analysis of the CRISPR/Cas system, many Cas variants have been developed for improved editing efficiency and performance [128]. After successful application of SpCas9 as a tool for genome editing in mammalian cells, other Cas9 proteins have been developed. For example, SaCas9 (*Staphylococcus aureus*) [129], Nme2Cas9 (*Neisseria meningitides*) [130], AsCpf1 (*Acidaminococcus sp*), LbCpf1 (*Lachnospiraceae sp*) [49,131], and recently identified AacCas12b (*Alicyclobacillus acidoterrestris*) [132] have shown comparable or higher genome editing efficiency to that of SpCas9 [128]. Furthermore, a recently reported type VI CRISPR-associated RNA targeting Cas13a and Cas13b system can specifically cleave strands of RNA [133–135].

Recent technological advances have enabled development of nuclease-deficient, catalytically-inactive dead Cas9 (dCas9) or paired nickase (Cas9N) [136] that can be fused with various transcription regulatory domains creating either CRISPR activator (CRISPRa) or CRISPR inhibitors (CRISPRi) that can specifically activate or silence the target gene [137]. In addition, the recently reported D10A cas9 nickase-based cytidine base editors (CBEs) and adenine base editors (ABEs) can generate A to T and C to G transitions, respectively, causing many double-stranded breaks in the targeted genomic region [138]. In addition, the Liu group developed high-efficiency H840A Cas9 nickase-based prime editors (PEs) designed to restore both strands to the desired sequence instead of the original without DSB or donor DNA template [139]. Thus, application of the CRISPR/Cas system, which can modulate target gene expression or transcript levels in a PAM-

independent and transient manner, could provide a controllable approach for disease modelling and treatment.

3. Development of models for CRISPR-based correction of defective genes

The CRISPR/Cas9 system has been widely used to create mutations in target genes and generate sequence-specific genomic modification through HDR. CRISPR has been used for other purposes such as regulation of endogenous gene expression, live-cell imaging of chromosomal loci, epigenome engineering, and high-throughput screening and editing of RNA [137]. Given its precision, simplicity, and robustness, CRISPR has emerged as a tool for generating novel *in vitro* and *in vivo* disease models and has the potential to connect biological discoveries with clinical therapeutic approaches. In this section, we review the use of CRISPR/Cas9 systems to develop various disease models.

3.1. Pluripotent stem cell technology: Toward gene correction in humans

The technique of culturing cells in a dish has been the backbone of basic biomedical research for decades because it can provide insights into normal and pathological cellular processes. Regenerative medicine and cell therapy aim to replace unhealthy or diseased cells with healthy new cells. Primary cells also can be genetically manipulated and re-implanted into patients to cure or treat genetic diseases [140]. However, the limited lifespan of primary human cells in culture is a drawback that limits inquiries into regulation of tissue formation, regeneration, and repair [141]. Most human cell lines used today have been derived from tumors or transformed derivatives of native tissues that carry mutations; the inability to faithfully adapt these human cells for *in vitro* growth limits research [141,142]. However, advances in reprogramming human somatic cells into induced pluripotent stem cells (iPSCs) have begun to address correction of defective genes in mammals.

Pluripotent stem cells (PSCs) or typical embryonic stem cells (ESCs) derived from the inner cell mass of blastocysts can differentiate into specialized cells to recreate specific tissues such as lungs or brain [143]. Thus, PSCs are an immortal population that can differentiate into virtually any cell type within the human body and enable establishment of new models of mammalian development and a new source of cells for regenerative medicine [144,145]. The immortality of PSCs could help to generate clinically relevant cell populations for aid in tissue repair and regenerative medicine, although they show difficulty in efficient genome editing [143]. On the contrary, iPSCs are easier to manipulate, but their applications in regenerative medicine approaches should be considered prior to human studies [146]. Issues such as immune rejections, carcinogenicity, genomic instability, lack of in situ integration and lack of quality controls have hindered application of iPSCs to routine clinical use [146]. However, a recent study published in a reputed journal has used autologous patient-derived iPSCs to dopaminergic progenitor cells to treat Parkinson's disease. Although this study is still in its infancy, it opens up numerous avenues toward application of iPSCs to treat human diseases [147].

iPSCs have made progress toward modeling of numerous human genetic disorders. The scope of CRISPR/Cas9 gene editing systems has been expanded to disease-focused research through production and characterization of patient-derived iPSCs that carry specific genetic diseases. CRISPR/Cas9 editing systems thus allow either repair of genetic mutations in patient-derived iPSC models or generation of disease mutations in healthy iPSCs to generate disease models. An example of the application of iPSCs toward modeling of human diseases was described for the multifactorial neurodegenerative Parkinson's disease (PD) [148]. Some PD cases are due to autosomal dominant mutations in *SNCA* genes that encode α-synuclein responsible for synaptic transmission and vesicle transport. Arias-Fuenzalida et al. generated isogenic-biallelic mutations in the *SNCA* gene using healthy iPSCs and FACS-assisted CRISPR/Cas9 editing to develop a PD-specific model [148]. CRISPR/Cas9 also has been used to derive isogenic *CSB/ERCC6* gene corrections in iPSCs derived from a rare autosomal recessive disorder cockayne syndrome (CS) patient [149]. Another recent report regarding the use of CRISPR/Cas9 to correct gene defects was demonstrated in amyotrophic lateral sclerosis (ALS), which is commonly caused by *C9orf72* mutations. CRISPR/Cas9-mediated excision of *C9orf72* repeat expansion reversed the pathophysiological effects of astrocytes in human iPSCs harboring these gene mutations [150].

iPSCs have the capability to differentiate into certain cell types relevant to disease phenotypes [151,152]. This differentiation capability make iPSCs an important asset to model numerous diseases, as described in the case of type 1 diabetes (T1D) by Leite et al. [153]. Autoimmune T1D results in destruction of pancreatic β cells that secrete insulin. Leite et al. used patient-derived endocrine cells and autologous immune cells to model T1D using an *in vitro* platform. They demonstrated a cell-type specific immune response against iPSC-derived β cells and a reduced effect against α cells [153]. Leite et al. demonstrated use of CRISPR/Cas9 systems to ablate HLA class I expression in iPSC-β cells, which resulted in reduced T cell activation, the significance of which has been described in the following sections (Section 4). iPSC-derived neurons also have been recently used to generate a CRISPR interference-based platform to study essential/non-essential gene functions [155]. Disease-in-a-dish *in vitro* iPSC models have been developed for numerous diseases including Shwachman-Bodian-Diamond syndrome [141], adenosine deaminase deficiency-related severe combined immunodeficiency [141], Duchenne and Becker muscular dystrophies [141,154], Gaucher disease type III [141], Parkinson's disease [141,156,157], Huntington's disease [141,158], Alzheimer's disease [159], rheumatoid arthritis and osteoarthritis [160], types 1 and 2 diabetes [141,153,161], the carrier state of Lesch-Nyhan syndrome [141], and Down syndrome/trisomy [141].

These disease models could also be used for high-throughput screening of therapeutic drugs. Advances in genotyping and next-generation sequencing techniques have facilitated identification of genetic mutations responsible for disease pathology and phenotypes [162]. Drugs elicit different responses in different patient-derived iPSCs, probably due to the different genetic backgrounds of individual donors [163]. Derivation of diseases models by inducing specific mutations in healthy iPSC cell lines (isogenic controls) allows identification of causative lesions that define cellular phenotype, resulting in disease. Such comparisons are not possible by comparing iPSCs-derived from patients and healthy individuals due to the inheritance patterns of single-nucleotide polymorphisms (SNPs) among individuals [162,163]. In addition to establishment of disease models without needing patient samples to screen for drug recovery efficacy, this strategy of disease modeling might be used to model various genetic conditions for which obtaining patient samples is problematic. The application of CRISPR-mediated gene correction strategies in pluripotent cells could confirm validity and safety before clinical applications while eliminating the need for murine models for validation. Disease-specific pluripotent cells capable of differentiating into the various tissues affected by each condition would provide insights into the pathophysiology of disease in a controlled, *in vitro* human system and allow screening of potential drugs in that system.

Nonetheless, certain challenges in the use of iPSC disease models need to be addressed. For example, the rarity of certain genetic diseases limits the ability to build patient-derived cell models, and genetic variations among populations could result in inappropriate interpretations of disease phenotypes *in vitro* [141,164,165]. The number and use of control cell lines during iPSC development, nuclease design, disease modeling, and drug screening also are controversial. Previously, the use of control cells from healthy family members was considered adequate, but isogenicity and genome sharing among family members could influence cellular disease phenotypes [166]. However, this problem can be tackled by the use of gene editing techniques to generate disease models in iPSCs derived from healthy individuals and eliminates the genetic modifiers that differ among patient-derived iPSCs and controls [162]. Another issue regarding use of iPSCs as disease models is the somatically acquired mutations in disease-affected cells, contributing to disease pathologies. Such mutations may not be present in iPSCs or iPSC-derived cells, though unrelated somatic mutations that have accumulated in cells used for reprogramming also would be propagated to iPSCs [162,167]. Advances in single-cell and deep-genome sequencing techniques can be utilized to study the effects of such mutations in diseases. Another issue regarding use of iPSCs arises due to the high clonal diversity of human iPSCs. Patient-derived iPSCs may not always be easy to derive, handle, or transfect; thus, protocols have to be adjusted constantly for individual cell lines. This may not be required when using universal human iPSC cell lines [166].

3.2. Transgenic animal models for in vivo gene editing

The CRISPR system has evolved at a rapid pace; during the past few years, its applications have expanded far beyond DNA mutagenesis [168]. Heritable gene editing has been achieved using the CRISPR/Cas9 system and generated a variety of transgenic animals. Those studies delivered the CRISPR/Cas9 system directly into fertilized zygotes using methods such as microinjection and electroporation incorporating DNA [169,170]. However, application of CRISPR to somatic tissues in postnatal animals has been challenging due to larger transgene size and existing delivery strategies for Cas9 *in vivo* [129]. To address that issue, various vectors, such as adeno-associated virus, lentivirus, and particle-mediated delivery, have been developed to offer low immunogenic potential, broad serotype specificity, and reduced oncogenic potential and host-genome interactions with the delivery of Cas9 [171,172]. Direct injection of nuclease-encoding messenger RNA into early mammalian embryos has proven quite effective in generating germline modifications, adding another tool for mammalian genetics [173].

CRISPR/Cas9 genome engineering has been successfully applied in many model organisms, including mice, cynomolgus monkeys, rats, pigs, *Caenorhabditis elegans*, *Xenopus tropicalis*, and *Drosophila* [169,174]. Among them, the mouse model is the most popular and has been used in developing many nuclease-directed systems, including CRISPR/Cas9, and brings a new level of flexibility to genomic manipulation [168]. The collective research using CRISPR/Cas9 for *in vivo* gene therapeutics in animal models of various human genetic diseases has demonstrated the potential applicability of the system for human use. These research advances suggest that CRISPR/Cas9 could be used to correct disease-driving mutations *in vivo* [164,169].

CRISPR/Cas9 systems have been routinely used to generate single-nucleotide polymorphism (SNP) animal models of human disease in rodents [169,175,176]. Such models provide a functional insight into human genetics that allow development of potential new therapies. An example of a pathological SNP modelled in mice was identified by human genome-wide association studies (GWAS) in the STXBP5 gene (rs1039084 A > G). STXBP5 acts as a regulator of platelet secretion in humans. The SNP-rs1039084 A > G has been reproduced by CRISPR in the mouse to display nearly the same thrombosis phenotype, confirming the causality of this SNP in humans [169,177].

The most popular application of CRISPR/Cas9 to the study of gene functions and drug discovery has been generation of specific gene knockouts. Conditional transgenesis in adult mice has been attempted to enable tissue-specific and inducible Cas9-mediated mutagenesis. Cre-mediated systems have been modified by including CRISPR targeting systems to enable them to express Cas9 under control of strong promoters such as CAGs [178]. Doxycycline-inducible systems have also been attempted, as demonstrated recently by Dow et al., to provide Cas9 and sgRNA to the germlines of animals. These models can target individual and multiple tissues and have not been restricted by the exogenous sgRNA delivery efficiency required to extinguish the expression of Cas9 after gene editing [179]. CRISPR systems also have been successfully utilized to insert *LoxP* sites surrounding various exons in mice. This strategy was described as *Easi*-CRISPR (Efficient additions with ssDNA inserts-CRISPR) that efficiently allows creation of conditional knock-in as well as knock-out models [180].

CRISPR also holds great promise for use in larger vertebrate model systems that reflect human disease progression better than mouse models. For example, pigs closely resemble human physiology and can be a highly relevant model for human diseases, as demonstrated by research into familial adenomatous polyposis and cystic fibrosis (CF). The existing mouse models of these monogenic diseases did not accurately resemble disease progression or treatment, and the pig models were more accurate. CRISPR can be applied to create monoallelic and biallelic mutants in higher animal models such as pigs, providing a gateway for improved study of cardiovascular diseases, metabolic treatments, and wound-healing. With implementation of new tools and technologies, these models could improve future drug discovery and treatment research [181,182]. Homozygous Pink1/Parkin knockout pigs have been developed as models for PD using CRISPR/Cas9 systems and somatic cell nuclear transfer (SCNTs) producing gene-edited animals with single identical mutations [183]. CRISPR/Cas9-based genome editing strategies have heightened the capacity to genetically modify pigs to engineer organs for xenotransplantation [184]. Toward such goals, CRISPR/Cas9 has been utilized to inactivate copies of the porcine endogenous retrovirus (PERVs) in pigs that could potentially be passed to humans [185]. Wu et al. demonstrated disruption of pancreatogenesis in pig embryos utilizing the CRISPR/Cas9 system to target the *PDX1* gene. These models could serve as a platform for xeno-generation of human organs in pigs when combined with chimeric-competent human iPSC technologies [186].

Another advantage offered by CRISPR/Cas9 is its ability to target multiple genes simultaneously, offering a way to analyze multiallelic mutants in a single step and removing the need to breed independent strains [168]. An example of this strategy was demonstrated by Li et al., who generated GGTA1/iGb3S and GGTA1/CMAH double knockout and GGTA1/iGb3S/CMAH triple knockout pigs that are naturally deleted in humans using the CRISPR/Cas9 system, indicating potential for xenotransplantation [187]. Multiple double-stranded breaks in DNA can also lead to genomic structural variations (SVs). SVs are large structural differences in genomic DNA that lead to chromosomal duplications, deletions, inversions, insertions, translocations, or combinations of these. Numerous human disorders are associated with such SVs involving several genes, and CRISPR/Cas9 systems have facilitated a way of modeling these diseases in mouse models [188–191]. Application of CRISPR/Cas9 to model human diseases containing SVs has been demonstrated for Downs syndrome using a

CRISpr MEdiated REarrangement (CRISMERE) strategy [188]. However, a drawback of targeting multiple sites using CRISPR is the sequence variations caused at breakpoints due to the randomness of NHEJ and the accessibility of CRISPR target sites that affect sgRNA efficiency [190]. CRISPR/Cas9 has been adapted for direct *in vivo* delivery systems that enable specific, multiplexed gene modifications in select mouse tissues. Proof-of-concept therapeutic models of CRISPR have been delivered via mosaic delivery systems that disrupt or modify target genes in subsets of cells that surround normal tissues [192]. CRISPR/Cas9 offers surgical-precision editing that leaves behind little or no trace of the entire process [192]. The future of genome editing could lie in successful, direct application of CRISPR *in vivo*, which could have multiple applications in precision medicine.

3.3. Organoid-based models offer futuristic approaches to gene correction

Organoids are 3D arrangements of specific cell types derived from stem cells that self-organize spatially and have restricted lineage commitments to represent the key structural and functional properties of organs [193]. Organoids that represent multiple niche interactions have been successfully established from adult stem cells and PSCs derived from mice and humans for multiple organs, such as the esophagus, salivary gland, prostate, fallopian tube, brain, liver, kidney, stomach, intestine, ovary, lung, and pancreas [194,195]. Technologies such as adeno-associated viral vectors and other lentiviral expression systems allow for genetic manipulation of organoids to successfully develop gene therapy strategies and disease models [196].

Because the genetic signatures of tissues can be retained in organoids, several genetic disorders, such as the autosomally recessive CF disorder caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, have been successfully modeled using organoids [195,197,198]. Schwank et al. demonstrated the successful application of CRISPR/Cas9-mediated correction of mutations in the CFTR regions of organoids derived from the intestines of two CF patients via homologous recombination [198]. The genome-edited organoids showed corrected CFTR gene expression with fully functional protein expression, indicating that CRISPR/Cas9 is a potential gene therapy strategy [198]. CFTR gene correction using CRISPR/Cas9 has been demonstrated in patient-derived iPSCs [199]. Organoids derived from CF patients have been deposited into biobanks and could have therapeutic applications in the near future [194].

Differentiated kidney organoids have been used to model congenital kidney disorders following introduction of disease mutations in human PSCs (hPSCs). This strategy was demonstrated by Freedman et al., who generated an *in vitro* human model of polycystic kidney disease (PKD) [201]. They used CRISPR to generate biallelic mutants of the PKD1 and PKD2 genes, which encode polycystin-1 (PC1) and polycystin-2 (PC2), respectively. These knockout cell-derived kidney organoids formed cysts that are characteristic of the disease in kidney tubules *in vitro* [201]. Genetically edited, podocalyxin gene-mutated hPSC cell lines have been developed using this strategy. Those gene-defective kidney organoids displayed junctional organization defects in podocyte-like cells, recapitulating glomerulopathy-like phenotypes *in vitro* [201].

Other kidney diseases have been studied in H9 human embryonic stem cells (hESCs) that were edited using CRISPR/Cas9 to target the mutations in the PKD1/PKD2 genes that cause PKD and autosomally dominant PKD [201]. The kidney organoids derived from PKD1 or PKD2 KO cells displayed about 6% cyst formation compared with wildtype kidney organoids, which resembles the clinical symptoms of PKD [202,203]. The application of CRISPR/Cas9-modified organoids has been extended to model diseases such as dyskeratosis congenital disease [204], monogenic diabetes [205], microcephaly [206], autism spectrum disorders [207], multiple intestinal atresia [208], and microvillus inclusion disease [209].

Therefore, organoids have great potential in cancer research, regenerative medicine, disease modeling, and drug screening, and they are a potential source of cells or whole organs for transplantation [210]. Combining iPSC generation strategies with CRISPR/Cas9-HDR gene correction and organoid generation would offer a personalized approach to correcting even rare genetic disorders and organ transplantation. Though use of iPSCs in regenerative medicine is currently hindered by various drawbacks, they hold great promise in this field. Numerous strategies are being investigated to aid generation of "safe" iPSCs that could have a positive influence on regenerative medicine [211,212].

4. Stem cell immune engineering to overcome HLA barriers in regenerative medicine

Despite the progress in regenerative therapies to replace or restore dysfunctional cells or tissues, immuno-incompatibility is a major barrier to clinical application. A host immune response called graft-versus-host disease (GVHD) can be triggered by the highly polymorphic human leukocyte antigen (HLA) system upon transplantation of cells or tissues into immune-competent individuals [212]. GVHD is triggered by alloreactive T-cells, which are the main cellular mediators that recognize non-self HLA molecules on the surface of allogeneic cells by a process called allorecognition [213]. Allogeneic donor cells can trigger the adaptive immune system via B lymphocytes, T lymphocytes, or natural killer (NK) cells [214,215]. CRISPR/Cas9 systems used with iPSC technology could establish universal donor iPSC cell banks to match the diversity of HLA phenotypes. The differentiation of such gene-engineered-iPSCs into desired cell types could be used as a strategy for regenerative medicine [216].

The HLA genes belong to the major histocompatibility complex (MHC) group of membrane-bound glycoproteins that comprises three classes, though MHC class III molecules are not involved in immunogenicity [215]. MHC class I genes are expressed on the surface of all nucleated cells and platelets and comprise three major genes, HLA-A, HLA-B, and HLA-C [217]. The polymorphism of MHC class I genes has been attributed to an alpha heavy chain, and its surface expression requires β2-microglobulin encoded by the *B2M* gene. These class I genes enable elimination of cells expressing foreign or viral antigens by presenting intracellularly processed peptides to $CD8^+$ cytotoxic T cells [218,219]. MHC class II genes include HLA-DR, HLA-DP, and HLA-DQ and are expressed only on specialized antigen-presenting cells such as macrophages, dendritic cells, and B cells. Non-self-antigens presented by MHC class II molecules trigger immune responses via $CD4^+$ T helper cells. The class II proteins contain polypeptide α and β chains and require the CIITA transcription factor to activate HLA II gene expression [218,219]. HLA homozygosity at HLA-A, -B, -C and -DRB1 plays an important role during transplantations at allelic levels. HLA homozygous haplotypes have been defined as having only one allele at each of these four loci, whereas HLA heterozygosity is defined as two alleles at any of these loci [220].

Many groups have attempted to achieve a universal donor using different strategies. Early strategies to achieve successful engraftment included identification of HLA homozygous iPSC cell lines at HLA-A, HLA-B, and HLA-DR loci [221–223]. Those homozygous cell lines, however, need to be matched at the HLA C1/C2 ligands to prevent attack by natural killer (NK) cells [224]. NK cells trigger an allogenic response when they do not detect HLA class I expression on cell surfaces through two inhibitory receptors, the killer cell immunoglobulin-like receptor (KIR) and NKG2A receptor

[224]. Precise CRISPR-based gene editing strategies have been used to efficiently target the exons of the MHC class I HLA-A, HLA-B, and HLA-C genes [225]. Various strategies have reduced HLA class I gene expression through knockout of *B2M* [226–230]. However, those cells could be targeted by host NK cells due to their deficiency of class I genes. Immune responses triggered via NKG2A receptors can be suppressed by HLA-E surface expression fused to the *B2M* promoter [228]. However, HLA-E overexpression does not suppress KIRD2$^+$ NK cells. The KIR2DL receptor requires HLA-C or HLA-G expression, as demonstrated by Han et al., who ablated the expression of HLA-A, HLA-B, HLA-C, and HLA class II genes in human iPSCs [224,228,231]. The HLA-deficient cells were modified to express HLA-G and the immunomodulatory factors PD-L1 and CD47, which resulted in a blunted T cell response [231]. A recent study has demonstrated generation of hypoimmunogenic human PSCs (hPSCs) by knocking out *B2M* to ablate HLA-class 1 and replace it with HLA-G1 within the frame of *B2M*. These engineered hPSCs are reported to be compatible with CD8 + T cells and NK cell-mediated immunotoxicity [232]. HLA-A and HLA-B carry Bw4 motifs that can be recognized by the KIR3DL1 receptors of NK cells [224]. HLA-C(C1) can suppress KIR2DL2/3 receptors, and HLA-C (C2) suppresses KIR2DL1 receptors [233]. On this basis, Xu et al. demonstrated that sequential deletion of HLA-A and -B in iPSCs using CRISPR/Cas9 while retaining expression of HLA-C allowed cells to evade CD8$^+$ T cells and NK cells more efficiently than *B2M* KO [234]. However, minor histocompatibility antigens on those MHC-edited cells could trigger snap responses in patients, diminishing the T cell repertoire [235].

HLA class II proteins present peptides to CD4$^+$ T helper cells and have been manipulated in ways similar to those described for class I genes. The CIITA transcription factor is essential for class II gene expression, and several studies have attempted CIITA KO using

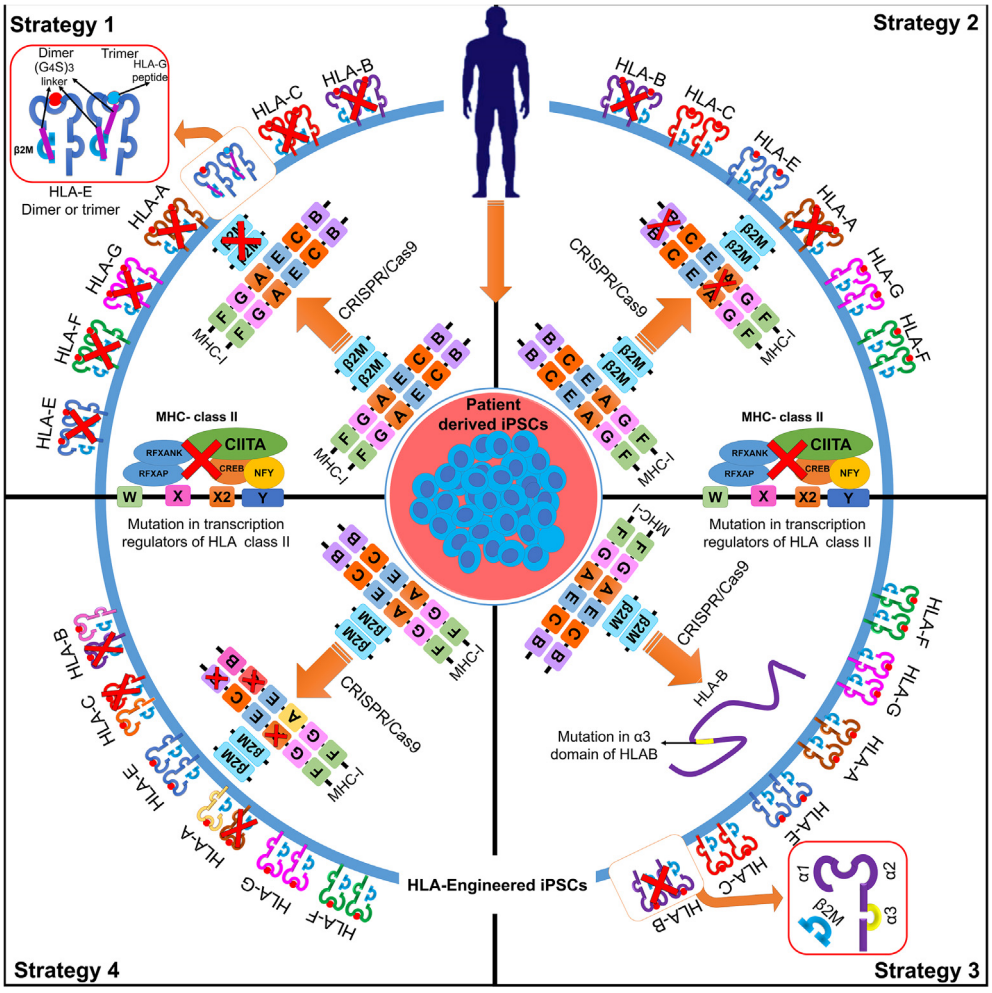

Fig. 2. CRISPR-based HLA immunoengineering toward development of immunocompatible-induced pluripotent stem cells. The figure represents various strategies aimed towards generation of HLA-immunocompatible iPS cell lines by selectively targeting HLA class I and/or II using the CRISPR/Cas9 system. Strategy 1: Selective knockout of transactivators for HLA class I-B2M gene and HLA class II-CIITA using a CRISPR/Cas9 system prevents immune response from cytotoxic CD8$^+$ and CD4$^+$ T cells [229]. However, lack of HLA surface expression can activate NK cells, risking proliferation of infected cells and increasing the risk of cancer. Exogenous surface expression of HLA E single-chain dimers fused to B2M promoter or as trimers containing HLA G signal peptides in cells lacking surface expression of HLA-A, B or C escapes allogenic recognition by CD8$^+$ T cells while conferring resistance to NK cell mediated lysis [236]. Strategy 2: HLA-C plays a pivotal role in suppressing activation of NK cells. CRISPR/Cas9-mediated disruption of HLA-A and HLA-B biallelically, while retaining HLA-C along with other non-canonical HLA class I in iPSCs expands donor compatibility while suppressing T cell and NK cell activation. The immunocompatibility of HLA-C-retained iPSCs can be improved by targeted knockout of class II transactivator-CIITA, suppressing activation of CD4$^+$ T cells [234]. Strategy 3: The CRISPR/Cas9 system has been demonstrated to increase immunocompatibility by knocking out heterozygous HLA-B in homozygous HLA-A iPSCs with an HLA haplotype HLA-A (24:02, 24:02) and HLA-B (40:01, 54:01). HLA-B-engineered iPSCs were generated by specifically mutating the alpha 2–3 domains that form the B2M binding groove. The HLA-KO homozygous iPSCs exhibit less immunogenicity while maintaining pluripotency [237]. **Strategy 4:** Generation of pseudo-homozygous iPSCs using the CRISPR/Cas9 system. HLA homozygous cell line stocks have been proposed to match a major percentage of the population [226]. CRISPR/Cas9 gene editing can be used to generate class I HLA haploid iPSCs by targeted allele-specific HLA disruption in HLA heterozygous iPSCs to generate "pseudo-homozygous" iPSCs with similar donor potential to HLA homozygous iPSCs [234].

CRISPR/Cas9 [228,231,234]. Deuse et al. generated HLA-I and HLA-II KO mouse and human iPSCs using CRISPR/Cas9, generating hypoimmunogenic iPSCs that could evade immune rejection [228]. However, depending on the cell fate of those modified stem cells, deletion of important HLA genes could affect antigen presentation. For example, dendritic cells derived from CIITA-deleted iPSCs could have ablated antigen-presenting functions. Various strategies aimed toward generation of universal donor stem cells are highlighted in Fig. 2.

The use of genome editing tools such as the CRISPR/Cas9 system with gene knock-in strategies opens avenues that could theoretically change the serotype of any HLA genes to better match donors to recipients. This strategy could potentially generate fully functional universal donor cell lines that would enable transplantation into any patient.

5. Transition of a CRISPR/Cas9-based genome editing system to the clinical system

CRISPR/Cas systems have applicability far beyond the disease modeling and universal cell line generation strategies reviewed in this article. Advanced clinically approved gene editing strategies rely on *ex vivo* gene manipulation that can provide therapeutic advantage following transplantation of modified cells into recipients. Currently, clinical protocols are in progress to study application of CRISPR/Cas9 technology in lung, renal, bladder, and esophageal cancer [128]. In particular, a gene editing approach using engineered T cells overexpressing chimeric antigen receptors (CARs) has been successfully used in T cell-based immunotherapies [238,239]. Researchers have successfully used multiplex genome editing to generate CAR-T cells resistant to inhibitory programmed

Table 3
The CRISPR/Cas-based cell therapy in clinical trial. Data available at https://clinicaltrials.gov/

Clinical Trial Identifier	Trial name	Disease	Target gene	Interventions	Phase of study	Group	Status
NCT03745287	A safety and efficacy study evaluating CTX001 in subjects with severe sickle cell disease	Sickle cell disease	*BCL11A*	Autologous CD34$^+$ HSPCs	Phase I/II	Vertex Pharmaceuticals Incorporated, USA	Recruiting
NCT03655678	A safety and efficacy study evaluating CTX001 in subjects with transfusion-dependent β-thalassemia	β-thalassemia	*BCL11A*	Autologous CD34$^+$ HSPCs	Phase I/II	Vertex Pharmaceuticals Incorporated, USA	Recruiting
NCT03728322	iHSCs with gene correction of HBB intervention subjects with β-thalassemia mutations	β-thalassemia	*HBB*	CRISPR–Cas9-treated induced hematopoietic stem cells	Early phase	Allife Medical Science and Technology Co., Ltd, China	Not yet recruiting
NCT03872479	Single ascending dose study in participants with LCA10	Leber congenital amaurosis type 10	*CEP290*	Subretinal injection of Drug AGN-151587	Phase I/II	Allergan, USA	Recruiting
NCT03166878	A study evaluating UCART019 in patients with relapsed or refractory CD19 + leukemia and lymphoma	B cell leukemia, B cell lymphoma	*TCR* and *B2M*	CRISPR–Cas9 treated CD19-directed chimeric antigen receptor T (CAR T) cells (UCART019)	Phase I/II	Chinese PLA General Hospital, China	Recruiting
NCT03081715	PD-1 knockout engineered T cells for advanced esophageal cancer	recurrent or metastatic esophageal cancer	PD-1	Autologous T-cells	Phase II	Hangzhou Cancer Hospital, China	Completed
NCT02793856	PD-1 knockout engineered T cells for metastatic non-small cell lung cancer	Stage IV non-small cell lung cancer	PD-1	Autologous T-cells	Phase I	Sichuan University, China	Active, not recruiting
NCT03545815	Study of CRISPR-Cas9 mediated PD-1 and TCR gene-knocked out mesothelin-directed CAR-T cells in patients with mesothelin-positive multiple solid tumors.	Solid tumor, adult	*PD1* and *TCR*	CRISPR–Cas9-treated CAR T cell infusions (*ex vivo*)	Phase I	Chinese PLA General Hospital, China	Recruiting
NCT03399448	NY-ESO-1-redirected CRISPR (TCRendo and PD1) edited T cells (NYCE T Cells)	Multiple myeloma, melanoma, synovial sarcoma	*PD-1* and *TCR*	CRISPR–Cas9-treated autologous T cells (*ex vivo*), combined with chemotherapy agents	Phase I	University of Pennsylvania, USA	Terminated
NCT04037566	CRISPR (HPK1)-edited CD19-specific CAR-T cells (XYF19 CAR-T cells) for CD19 + leukemia or lymphoma	CD19-positive leukemia, lymphoma	*HPK1*	CRISPR–Cas9-treated autologous CD19-directed T cells	Phase I	Xijing Hospital, China	Recruiting
NCT03164135	Safety of transplantation of CRISPR CCR5-modified CD34 + cells in HIV subjects with hematological malignancy	HIV-1-infection	*CCR5*	Transplantation of CRISPR–Cas9-treated CD34 + HSPCs (*ex vivo*)	N/A	Academy of Military Medical Sciences, China	Recruiting
NCT03057912	Safety and efficacy study of TALEN and CRISPR/Cas9 in treatment of HPV-related cervical intraepithelial neoplasiaⅠ	HPV-related malignant neoplasm	HPV *E6/E7*, 16 and 18	CRISPR/Cas9-HPV18 E6/E7T2	Phase I	Sun Yat-Sen University	Not yet recruiting
NCT03855631	Exploiting epigenome editing in Kabuki Syndrome: a new route toward gene therapy for rare genetic disorders (Epi-KAB)	Kabuki syndrome 1	*KMT2D*	Unknown (*ex vivo*)	N/A	University Hospital, Montpellier, France	Active, not recruiting

cell death protein 1 (PD-1) that prevents recognition of tumor cells by T-cells [239–241]. In fact, knock out of PD-1 in autologous T-cells and infusion back into cancer patients was the first reported use of CRISPR/Cas9 system in a human clinical trial in Europe (NCT03399448) and China (NCT03081715) [242]. Several clinical trials registered at www.clinicaltrials.gov (NCT04438083, NCT03545815, NCT04037566, NCT04035434) are studying the safety and efficacy of CRISPR/Cas9-engineered T cells to treat cancer [243]. The first reported CRISPR treatment for a rare condition called Leber congenital amaurosis 10 (LCA10) used *ex vivo* correction of patient-derived retinal cells. The CEP290 gene mutation responsible for LCA10 was deleted using CRISPR/Cas9 in retinal cells that were then re-implanted into the eye [244,245]. Human trials based on the CRISPR/Cas system have been undertaken for treatment of various genetic diseases like β-thalassemia (NCT03655678) (NCT0372832) and Kabuki syndrome 1 (NCT03855631) [246] (https://clinicaltrials.gov/). Table 3 summarizes the application and advances of CRISPR/Cas system in clinical trials. Collectively, the success of these clinical trials suggests the potential of development and establishment of protocols for treatment of various diseases and cancer using a CRSISR/Cas9-based genome editing system.

6. Concluding remarks

CRISPR/Cas systems have been efficiently used to generate disease models, and great strides have been made toward clinical applications. Research evidence demonstrates that genome editing systems such as CRISPR/Cas9 can make significant contributions to therapeutic strategies for various human diseases by directly interfering with target genes or deriving multifunctional tools. The news of the first CRISPR-gene-edited babies in China back in 2018 shocked the scientific world. That work, conducted by He Jiankui et al., was a direct violation of the international scientific consensus that gene editing using CRISPR/Cas9 is still in its infancy and is not ready to use in germline modifications of humans, which could be passed through generations [247]. However, CRISPR technologies have progressed ethically toward clinical trials. Development of CRISPR/Cas9 genome editing technology has enabled therapeutic application of CAR-T therapy through targeted intervention of endogenous genes [248]. Engineered CAR-T cells called iCARTs can be generated from patient-derived iPSCs with HLA-independent customizable antigen recognition, and they have been shown to kill cancer cells [248–250]. Together, these studies show that patient-derived iPSCs or hESCs used to generate NK cells in combination with CRISPR/Cas9-mediated gene modification could be an efficient cancer immunotherapy. Such studies provide hope that CRISPR will advance as an off-the-shelf cell therapeutic approach.

Although hindered by drawbacks such as potential off-target effects, Cas9 immunity, and HDR efficiency for gene correction, this genome editing technology is progressing toward clinical application. Much research is being conducted to tackle such drawbacks; for example, the specificity of CRISPR can be improved by modifying Cas9 construction [251], optimizing sgRNA designs [97,252], or using Cas9 mRNA for transient Cas9 expression [44]. The efficiency of HDR has been improved utilizing single-stranded donors [253,254] and small molecules [14,16], varying the lengths of homologous arms [255], or using modified double-stranded donors [256]. The immunogenicity of CRISPR/Cas9 can be addressed utilizing non-viral delivery systems such as nanoparticles or lipid-based vectors [257,258]. Deeper exploration of this genome editing technology has the potential to uncover fundamental biological mechanisms that drive many diseases and provide permanent cures for previously untreatable diseases. The compatibility of CRISPR systems with various biological systems, including stem cells, makes them an incredible tool. However, use of stem cells is surrounded by controversies including the ethical issues surrounding embryonic stem cells (ESCs) and somatic cell nuclear transfer (SCNTs), carcinogenicity, and genetic instability factors surrounding iPSCs [146,259,260]. iPSCs are not hindered by ethical issues, and progress is being made toward derivation of clinically relevant "safe" iPSCs. Efforts are being made to replace the oncogenic genes in the reprogramming cocktail that typically include *Oct4, Sox2, Nanog,* and *c-Myc* with non-oncogenic genes such as *Nkx3.1* or by eliminating them completely [261–265]. Gene-corrected iPSCs could be differentiated into specific cell types, and such "safe" stem cell lines could be developed as universal donor stem cells that can be transplanted into any patient and can be realistically achieved using CRISPR systems to revolutionize the future of regenerative medicin.

CRediT authorship contribution statement

Ainsley Mike Antao: Conceptualization, Software, Writing - original draft. **Janardhan Keshav Karapurkar:** Conceptualization, Software, Writing - original draft. **Dong Ryul Lee:** Writing - review & editing. **Kye-Seong Kim:** Supervision, Writing - review & editing. **Suresh Ramakrishna:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

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

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