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Review

CRISPR/Cas9 Technology in Translational Biomedicine

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Key Words
CRISPR • Cas9 • HR • NHEJ • MMEJ • Gene therapy • Animal models • Human disease

Abstract
The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) - RNA-guided Cas9 endonuclease system has provided a fast and efficient method for precise genome editing in diverse mammalian species, including humans. The CRISPR/Cas9 technology allows generation of modifications into site-specific locations of the selected genes in one major step by carrying deletions, insertions or DNA donor-directed precise sequence modifications. Cas9 forms a nucleoprotein complex with a sequence-specific guide RNA to create double-stranded breaks in complementary DNA target. Further, double-stranded break repair machinery leads to the intended gene modifications. The CRISPR/Cas9 system is widely used technique for genome modification, editing and other biotechnology applications, such as functional annotation, a system for visualization of specific genomic loci and transcriptional control of genes. CRISPR/Cas9-mediated manipulation of the laboratory animal genomes has contributed to the understanding of gene functions and has become a popular approach for modeling human disorders. Furthermore, the growing application of CRISPR-Cas9 system to human genes emerges as an extremely powerful technology for the molecular characterization and treatment of human disease. In this review we present the essential principles of CRISPR/Cas9 technology and the recent advances in its use in translational biomedicine.

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Introduction

System of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas) is known as a natural RNA-guided adaptive immunity system of bacteria and archaea that protects them against phages. CRISPRs were first identified in 1987 in DNA sequence from Escherichia coli [1]. Later this system was found in other bacteria and archaea [2–4]. In the early 2000s, three research groups independently discovered that some of CRISPR sequences have bacterial and archaeal viruses origin

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[5–7]. In the following years, the molecular mechanisms and functional characteristics of CRISPR/Cas systems were uncovered by comparative genomic and structural analyses [8]. In 2012, Jinek et al. demonstrated that Cas9 endonuclease can be programmed with single guide RNA(sgRNA) to bind target and make site-specific double-stranded breaks in any DNA sequence of interest [9]. In 2013, the system was applied to genome engineering in eukaryotic cells [10]. Since that time, the system is successfully used throughout the world for genome editing. Two classes of CRISPR/Cas systems have been identified: multimeric class 1 and monomeric Class 2. Class 1 is characterized by effector complexes that consist of multiple Cas proteins, whereas Class 2 effectors is recognized by a single, multi-domain Cas protein [11]. Both Classes use CRISPR RNAs to direct a Cas endonuclease cleaving its target double-stranded DNA (dsDNA) sequence. Due to their simplicity, Class2 CRISPR-Cas systems have been adopted for application in the field of genetic engineering of mammals and plants. Class 2 system has been grouped into 3 distinct types: type II -Cas9 (Csn1), type V-Cas12 (Cpf1 and C2c1), and type VI - Cas13 (C2c2). Interestingly, Cas13 is the only endonuclease of CRISPR-Cas systems that exclusively target RNA [12, 13]. We focused our review on the basic mechanism of type II CRISPR/CAS9 technology and discuss here recent progress in its applications in various fields of translational biomedicine.

CRISPR/Cas9: structure and mechanism

CRISPR/Cas9 system contains three major components: the RNA-guided endonuclease Cas9, CRISPR RNA (crRNA) and transactivation CRISPR RNA (tracrRNA). Structural studies of Streptococcus pyogenes Cas9 (SpCas9) have revealed a bilobed architecture composed of recognition lobe (REC) and nuclease lobe (NUC) [14–19]. The REC lobe, in its turn, consists from two domains: REC and Bridge Helix (BH). NUC lobe has three domains: protospacer adjacent motif interacting (PI), HNH and RuvC (Fig. 1). The REC lobe is responsible for sgRNA binding, while BH domain plays role in initiating cleavage activity right after binding DNA. The PI domain is responsible for binding protospacer adjacent motif (PAM) contributing to local strand separation of the target DNA duplex and sgRNA-DNA hybrid formation. All known Cas9 enzymes contain an HNH domain that cuts the complementary to the guide RNA target-DNA strand through a single-metal mechanism, and a RuvC nuclease domain that cleaves the non-complementary strand of target-DNA through the two-metal mechanism, working together they result in double-strand DNA breaks. It has been shown, that HNH domain has H-N-H (histidine-asparagine-histidine) motif, which is typical for many endonucleases and consists of two antiparallel β-strands connected and surrounded by an α-helix [20]. RuvC domain is also well known in homologous recombination in bacteria due to its resolvase activity [21]. The crRNA sequence can be split into two regions- guide (20-nt) and repeat (12-nt). At the same time, the tracrRNA sequence can be split into two regions - anti-repeat (14-nt) and three tracrRNA stem loops. To program sequence-specific Cas9 dsDNA cleavage in eukaryotic cells, the dual tracrRNA:crRNA were fused in single guide RNA (sgRNA) (Fig. 2). The high-resolution crystal structure SpCas9 in complex with sgRNA demonstrated essential steps of functional interactions that mediate the direct double-strand cleavage. Consequently, it has been shown that crRNA spacer interacts with target DNA protospacer. While spCas9 interacts with the tracrRNA through the sequence-dependent connections: stem loop 1 is identified by the REC, the BH and the PI domains; stem loops 2 and 3 are recognized by the NUC lobe and crRNA is identified by the REC and NUC lobes [16]. The spCas9 protein remains inactive (Fig. 3A) until it connects with sgRNA following by the extensive structural rearrangements of the inactive form into active one (Fig. 3B). As soon as spCas9 makes complex with sgRNA and became active, it interacts with DNA sequence that perfectly matches both the target complimentary region of sgRNA and DNA containing a PAM motif (NGG) (Fig. 3C). SpCas9 recognizes a 5′-NGG-3′ PAM located downstream of the cleavage site on the non-complementary DNA strand and generates "blunt ends" after 3 base pairs upstream of the PAM site (Fig. 3D) [15]. It has been demonstrated that both purine bases

Fig. 1. A. Schematic illustration of domain organization for the type II spCas9. B. Cartoon-surface representations of the three-dimensional structure of spCas9 ID 4CMP from the Protein Data Bank (PDB) database. Structural image was prepared with the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC and Adobe Illustrator.

Fig. 2. A. Schematic illustration of the sgRNA–target DNA complex - (N- any nucleotide DNA- crRNA targeting sequence, duplex crRNA:tracrRNA sequence, and tri-loop tracrRNA sequence). B. Cartoon-surface representations of three-dimensional structure of sgRNA ID 5Y36 from PDB database. Structural image was prepared with the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC and Adobe Illustrator.

(G and A) at PAM-proximal sgRNA position 20 appear to have a positive impact on spCas9 performance that increase genome editing efficacy [22]. Notably, the extending sgRNAs additional PAM-distal bases (21–30) does not contribute to overall targeting specificity of Cas9-mediate levels of modification [23]. To maximize SpCas9 efficiency, cytosine is desirable at the DNA cleavage site (−3 position) [24]. Once the DNA is cut, the only one from a number of different DNA repair systems makes correction, which can lead to desired gene modification. Interestingly, isolated from different species Cas9 endonucleases, in spite of structural similarities, can identify distinct PAM [25]. For example, Cas9 endonuclease from *Francisella novicida* (FnCas9) recognizes a 5′-YG-3' sequence (Y- pyrimidines), while Cas9 from *Staphylococcus aureus* (SaCas9) identifies a 5′-NNNNGATT. The Cas9 PAMs sequences

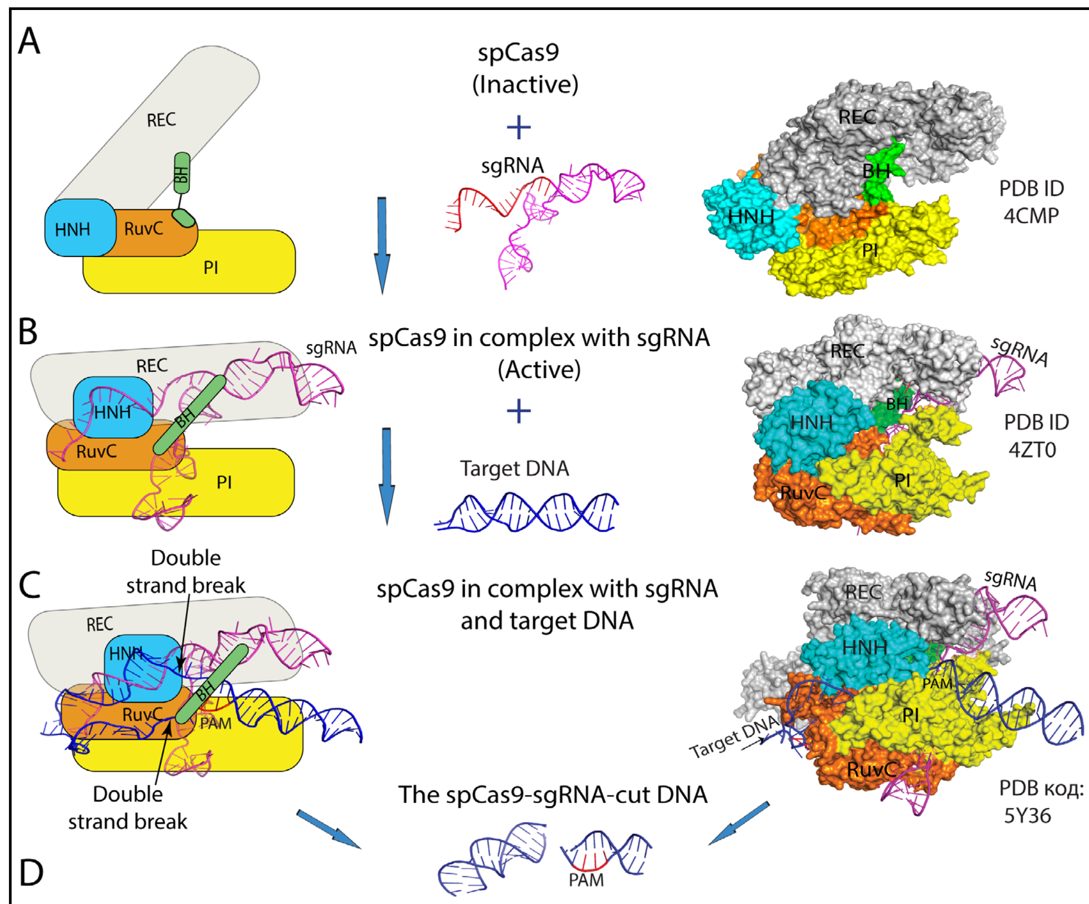

Fig. 3. A. Schematic illustration of spCas9 inactive form and surface representations of the three-dimensional structure of spCas9 ID 4CMP from PDB database. B. Schematic illustration of three-dimensional structure of complex spCas9 active form with sgRNA and its surface representations (ID 4ZT0 from PDB database). C. Schematic illustration of three-dimensional structure of complex spCas9 active form with sgRNA and target DNA and its surface representations (ID 5Y36 from PDB database). D. Cartoon representation of the spCas9-sgRNA-cut three-dimensional structure of DNA ID 5Y36 from PDB database. All crystal structural images were visualized with the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC and Adobe Illustrator.

from different species are listed in Table 1. Furthermore, the molecular mechanism of Cas9 for DNA cleavage at target sites directed by guide RNAs has provided a multiple platform for genome engineering. The distinctive feature of one-metal-ion-dependent nucleic acid cleaving enzymes is conserved two histidines, while for one-metal-ion-dependent nucleic acid cleaving enzymes a conserved aspartate residue is required [32]. This feature has been confirmed in several Cas9 mutagenesis studies resulting in producing various Cas9 variants, such as a nickase Cas9(nCas9) and a

Table 1. The Cas9 PAMs sequences from different species

Cas9 ortholog	PAM	Reference
Streptococcus pyogenes (SpCas9)	5′-NGG-3′ (N-any)	[9, 17]
Francisella novicida (FnCas9)	5′-YG-3′ (Y- pyrimidines)	[26]
Staphylococcus aureus (SaCas9)	5′-NNGRRT-3′ (R- purines)	[1]
Streptococcus thermophiles (St1Cas9)	5′-NNAGAAW-3′ (W- A or T)	[27]
Streptococcus thermophiles (St3Cas9)	5′-NGGNG	[28]
Neisseria meningitidis (NmCas9)	5′-NNNNGATT-3′	[29]
Neisseria meningitidis (Nme2Cas9)	5′-NNNNCC-3′	[30]
Lactobacillus buchneri (lbCas9)	5′-AAAA-3′	[31]

catalytically inactive dead Cas9 (dCas9). The conversion of Cas9 into a nickase form results in the ability of the CRISPR/nCas9 to make a cut of only one of the DNA double strands. To create nCas9 the histidine residue needs to be replaced by alanine in HNH domain (H840A) or the aspartate residue needs to be replaced by alanine in the RuvC domain (D10A) (Fig. 4). At the same time, mutating both nuclease domains of Cas9 abolishes endonuclease activity creating a dCas9. dCas9 can work as a site-specific DNA binding protein alone or can be combined with transcriptional activation or repression factors to control transcription.

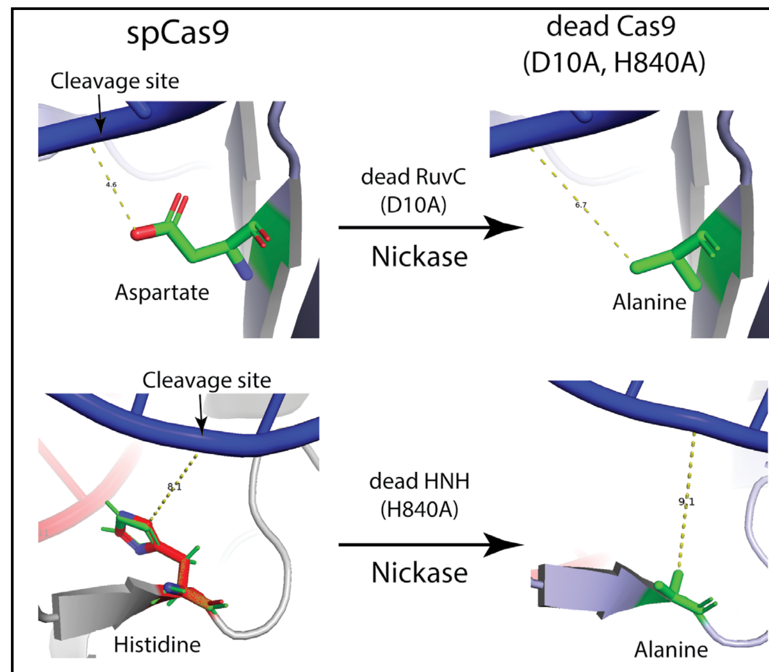

Fig. 4. Cartoon representation of the mutation spCas9- (PDB ID 5Y36) into nickases (D10A or H840A) and dead endonuclease (D10A and H840A). The Alanine residues were mutated to Asparagine and Histidine. The image was prepared using the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.

Overview of CRISPR/Cas9-mediated double-strand breaks repair machinery

DNA double-strand break is a dangerous lesion; however, it is common event during cell division and differentiation. Therefore, a number of proteins scan DNA to detect such lesions and, if recognized, activate cell repair machinery. There are three distinct pathways that are essential to repair Cas9- mediated double-breaks of DNA in mammalian cells: homologous recombination (HR); Ku-dependent non-homologous DNA end joining (NHEJ); and microhomology-mediated end joining (MMEJ). HR is termed to as «homologous» because a homologous sequence is needed to mediate the break ends repair. In contrast, NHEJ is named as “non-homologous” due to break ends direct ligation without the need of a homologous template. And finally, MMEJ referred as “microhomology” because of an alignment of microhomology sequences is required to complete break ends repair.

Molecular mechanism of Homologous Recombination (HR)

HR plays essential in meiosis and mitosis exchanging of genetic information between the donor and acceptor DNA [33]. HR-directed repair requires a homologous DNA template, that can be used for programming gene modifications. HR repair mechanism include five key steps: 1. recognition of double strand break; 2. 5’- to 3’- resection of broken ends; 3. finding the homologous sequence template; 4. fill-in synthesis and 5. ligation (Fig. 5). For recognition of double strand break, the complex of MRE11-RAD50-NBS1 (MRN) proteins is responsible. Right afterwards, CtIP nuclease contributes to the generation of 3′-end formation of single-stranded DNA (ssDNA). The ssDNA, in its turn, is bound by DNA replication protein A (RPA), which is further replaced by Rad51 with the assistance of BRCA2. BRCA2 plays essential

role in stabilizing RAD51 filaments and preventing MRE11-mediated degradation [34]. The Rad51 nucleoprotein filament performs homology sequence search and mediates strand invasion on the homologous template and extension of the displacement loop (D-loop). The capture of the second end leads to open template for synthesis a new string of DNA. The B family is formed by DNA polymerases α, δ, and ε that are essential for fill-in synthesis in HR repair pathway [35]. During the final step, strand invasion generates a Holliday junction, which can be resolved in either of two orientations and be ligated by DNA ligase I. It has been shown that HR rate can be different in accordance with the homology-arm length [36]. In mammal cells, HR is predominant during S (DNA replication) and G_2 (the growth) phases of the cell cycle [37].

Molecular mechanisms of Non-Homologous DNA End joining (NHEJ)

NHEJ process is accomplished by a series of proteins that work together to join DNA "blunt ends", following by terminal end processing and ligating (Fig. 6). The double-strand breaks are first recognized by the Ku70–Ku80 heterodimer (Ku), which forms a ring that surrounds two broken DNA 3'-ends that protecting them from degradation [38]. While Ku effectively binds the DNA, free access of polymerases, nucleases and ligases to the broken DNA ends remains open. Once Ku is in place, it recruits the catalytic subunit of a protein kinase DNA-PKcs to phosphorylate Artemis endonuclease and DNA ligase IV and XRCC4 complex (Fig. 5B). Artemis/DNA-PKcs complex is known to have 5'- and 3'- exonuclease activities resulting in removing uncoupled overhanding ends of DNA. Alternatively, single-stranded overhang requires polymerases to fill-in gaps by DNA synthesis before ligation. It has been identified that two polymerases from X family are involved into NHEJ: Pol μ (template-independent) and Pol λ (template-dependent) [39]. DNA ligase IV interacts with XRCC4 to accomplish the ligation step. The phosphorylation of DNA ligase IV and/or XRCC4 plays an essential role in their interactions with heterodimer Ku to promote ligase activity. NHEJ-mediated repair mechanisms can make small insertion, deletions or accurate

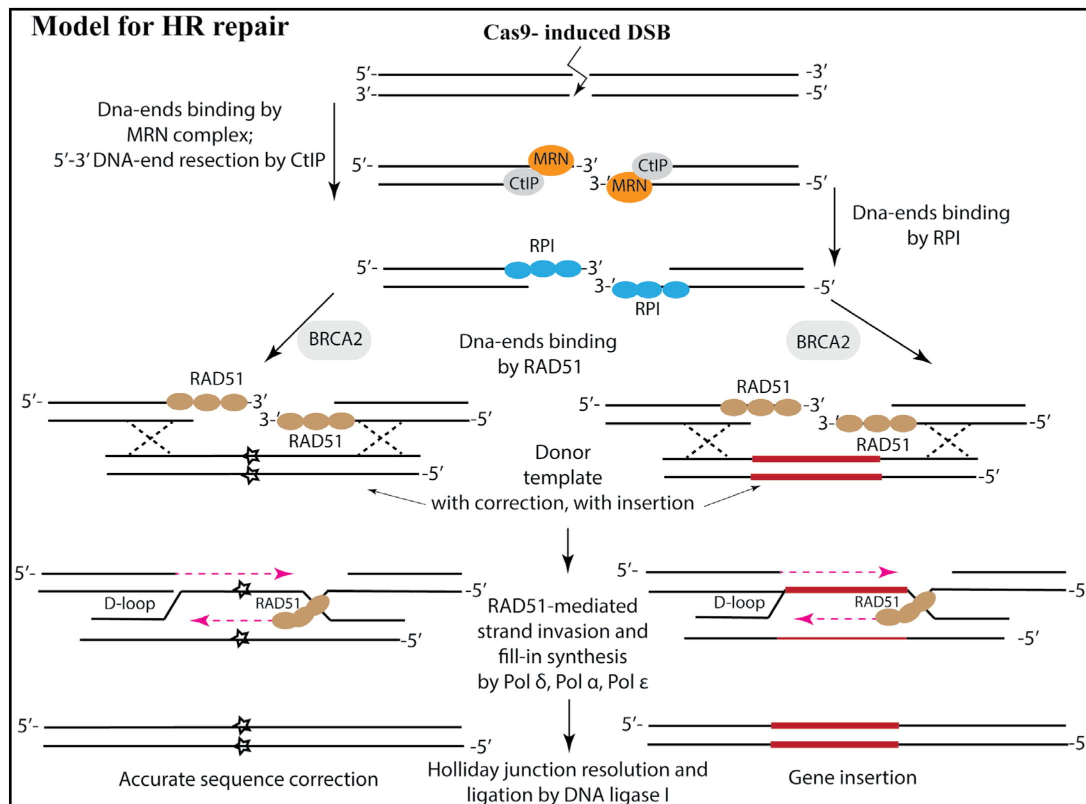

Fig. 5. Schematic illustration of HR repair model in mammalian cells (please see details in the text).

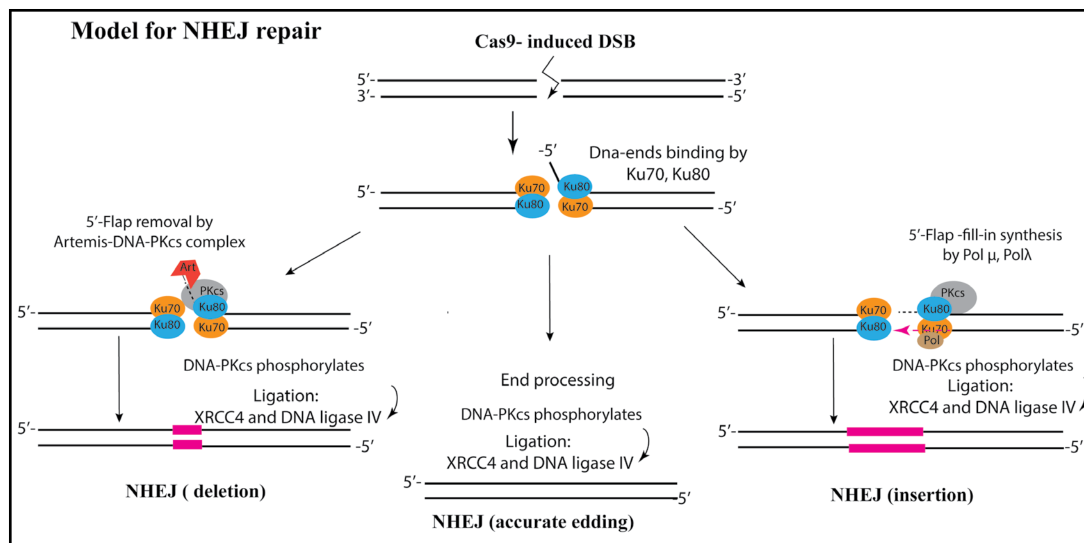

Fig. 6. Schematic illustration of NNEJ repair model in mammalian cells (please see details in the text).

correction of target DNA sequences. In eukaryotes, NHEJ is generally taken place in G_1 phase of the cell cycle and it is inhibited during mitosis [40].

Molecular mechanism of Microhomology-Mediated End Joining (MMEJ)

When NHEJ is absent owing to a lack of Ku or the DNA ligase complex, blunt ends can be joined by using MMEJ repair mechanism that involves alignment of microhomology sequences (within 5–25 bp range) flanking a double strand break. This process results in both deletions in between the homology arms or, in some cases, complex insertions (Fig. 7). The first end resection steps are similar in MMEJ and HR double strand break repair pathways [41]. Both HR and MMEJ pathway are initiated by DNA end resection with the help of MRN-CtIP complex (Fig. 5 and 7). The Mre11 nuclease activity is required for initial short-range resection at one break end. It has been shown that long range resection may inhibit MMEJ by activating HR repair pathway. One of the crucial factor for MMEJ repair is A-family DNA polymerase θ (Polθ) [42]. Polθ contains an N-terminal helicase and C-terminal polymerase domains separated by a flexible linker region. Notable, Polθ contains the Rad51 binding motif whereby it blocks RAD51 nucleofilament coating of 3′- formation of ssDNA, resulting in suppressing the HR repair pathway [43]. Polθ requires a short double-stranded DNA to start synthesizing pre-existing microhomologous sequences. The unwinding helicase activity serves to make the polymerase more processive. For flap removal the substrate structure specific endonuclease, such as XPF/ERCC1 is required. The final step of MMEJ is ligation by DNA ligase I or III with help of XRCC1 [44]. MMEJ seems to be most active during the M and early S phases in dividing cells [45].

The molecular mechanisms of preference in cellular repair post-CRISPR/Cas9 cleavage are not yet fully understood [46]. The choice what kind of repair pathway will be activated depends on many factors, such as the phase of the cell cycle, chromatin structure and the CRISPR/Cas construction [47–49]. For example, after resection the break can no longer be repaired by NHEJ. In this situation, the main competition will be between HR and MMEJ. However, it has been proposed that in mammals NHEJ is faster and more efficient [50].

CRISPR/Cas9-mediated modeling of human diseases in animals

CRISPR/Cas9 gene editing tool has created the opportunity to precisely modify genes in cell lines or animal models in order to study gene functions and molecular interactions underlying pathogenesis of various human diseases. Animal models of human disease are

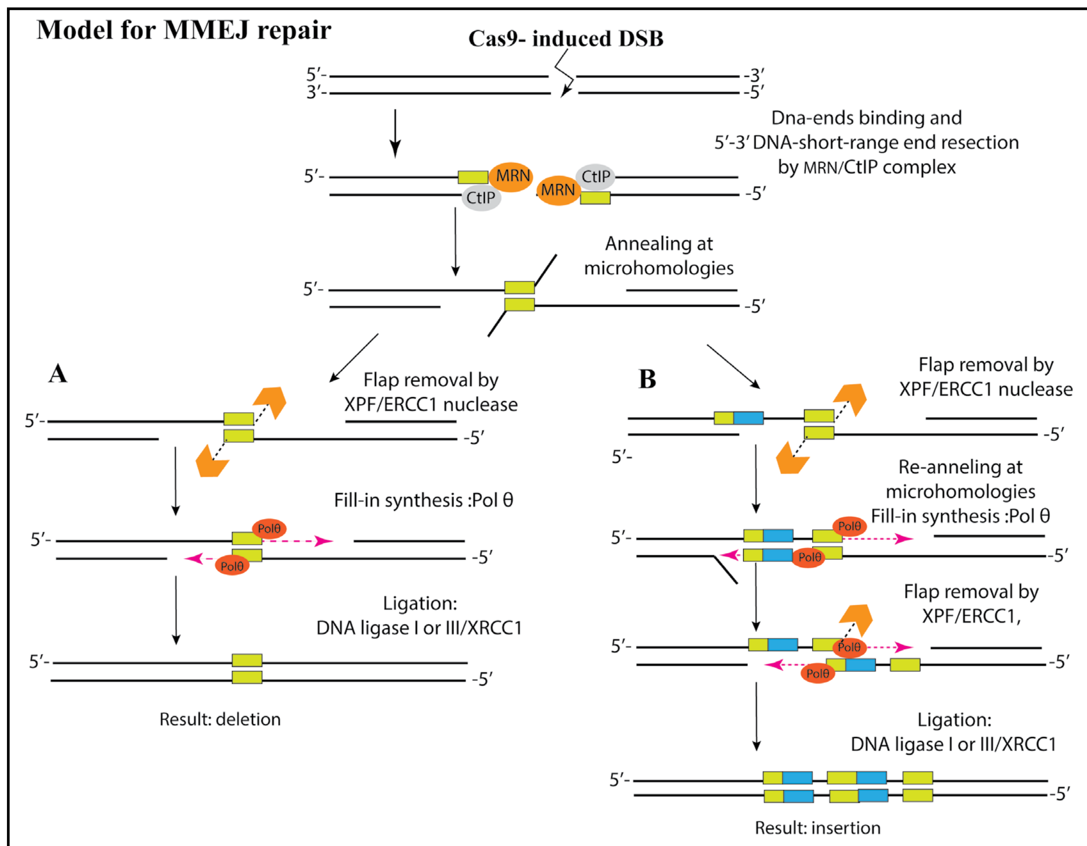

Fig. 7. Schematic illustration of MMEJ repair model in mammalian cells (please see details in the text).

crucial elements of drug development by providing *in vivo* test systems for evaluation of their potential efficacy in preclinical testing. Preclinical studies conducted on genetically modified animals represent one of the main sources of valuable information about the function and interaction of genes, identifying their association with a particular disease and translating the results obtained *in vivo* in animal models to humans. CRISPR/Cas9 technology provides a powerful genome-editing tool and has been successfully applied across various model organisms to quickly and efficiently create targeted genetic mutations. For example, it has enabled researchers to create new rodent mutant models in less than 4-8 months. In part, it is achieved by the direct cytoplasmic injection of Cas9 mRNA and adjacent single-guide RNA into mouse zygotes (alternatively an electroporation) followed by reimplantation into pseudo-pregnant females that can give a birth of genetically modified pups in a single step [51, 52]. Recently, the delivery of Cas9-sgRNA has been even more simplified due to self-delivery of the recombinant adeno-associated vectors (rAAV) into zygotes during co-incubating in KSOM culture medium or by direct transplantation of rAAV particles into the oviduct of pregnant females (Fig. 8B) [30, 53]. But, in any of these methods, the majority of gene-modified newborns show mosaicism, with mutation occurring only in a portion of cells. Therefore, several free bioinformatics tools have been developed to help in analyzing the first mosaic sequences, such as https://www.synthego.com/, https://tide.deskgen.com/ and others. To help in design of highly active and specific sgRNA, several programs has been also developed https://zlab.bio/guide-design-resources, most of them providing algorithms to predict on-target specificity of sgRNA and off-target side effects. For example, to identify potential off-target sequences in genome, the programs can suggest certain general design strategy. The activity of SpCas9, can be directed by the sgRNA with some mismatches in PAM-distal counterparts. High concentration of CRISPR/Cas9 constructs can increase the propensity of off-target effect. In addition, beside 5′-NGG-3′PAM identification, SpCas9 with

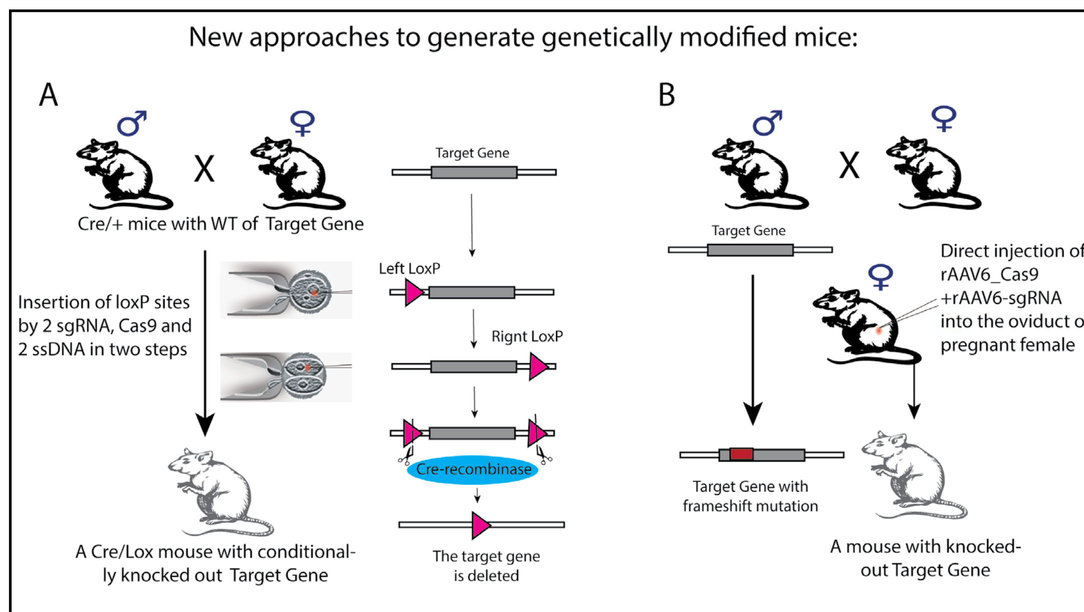

Fig. 8. Some of new approaches to generate genetically modified mice. A. FLP/FRT or Cre/LoxP site-directed recombination systems have been used to generate conditional knockout mutations. B. Direct transplantation of rAAV particles into the oviduct of pregnant females to generate knockout mice.

low level of efficiency can also recognize PAM 5′-NAG-3′ sequence, resulting in off-target cleavage of DNA [24, 54].

To knockout a protein coding gene, a CRISPR/Cas9 nuclease is usually designed to make double-stranded breaks in the beginning of the coding region of the gene of interest to mediate a frameshift mutation. As a rule, transcripts containing frameshift mutation are degraded by nonsense-mediated mRNA decay (NMD) [55]. Anyway, in some cases nonsense transcripts cannot be degraded by NMD resulting in translation of C-terminally truncated proteins, which could have unpredictable effect of cell function [56]. On the other hand, even small changes in target DNA, such as gene point mutations can result in strong phenotypes in model animals. Owing to these causes, it is important to know if the gene is completely knocked out or it continues to function and how this function differs from that in wild-type animals. Some mutations could be embryonic lethal and the only way to study the gene functions is creation the conditional knockout of target genes in adult mice. The methods that combine CRISPR/Cas and two classical Flp/FRT or Cre/LoxP site-directed recombination systems have been used to generate conditional knockout mutations [57, 58]. Isolated from yeast *Saccharomyces cerevisiae* recombinase Flippase (Flp) distinguishes a pair of the 34-bp sequence 5′-GAAGTTCCTATTCtctagaaaGTATAGGAACTTC-3′ (FRT- flippase recognition target), when P1 bacteriophage cyclization recombination (Cre) recombinase recognizes a pair of the 34-bp target sequence 5′-ATAACTTCGTATAatgtatgcTATACGAAGTTAT-3′ (loxP). The Flp and Cre recombinases gene expression can be under the control of the tissue-specific promoters. Both Flp and Cre recombinases can initiate cleavage, exchange of each strand, and ligation of target DNA flanked by two Frt or loxP sites, respectively. Therefore, Cas9-induced double-strand breaks are used to direct homologous recombination-dependent insertion of single-stranded DNA (ssDNA) template that contain FRT or LoxP sequences up- and downstream of the target gene. Usually, Flp and Cre strains are developed independently and then use them to implement the FRT or LoxP sequences (Fig. 8A). Interestingly, the location and orientation of the loxP sites can direct Cre recombinase action. Thus, it can mediate deletions (same direction), inversions (opposite direction), and translocations (located on different strands of DNA in same direction) of the floxed locus of target gene [59]. To induce recombinase-mediated DNA sequence exchange, both the template and

target DNA sequence are flanked by FRT or loxP. Most recently, the long single-stranded (lssDNA) donors DNA-based knock-in technique revealed higher targeting efficiency and this approach simplified whole process of disease model creation in comparison to traditional FRT/LoxP-mediated DNA sequence exchange approaches. Moreover, this technique enables the insertion of larger fragment of DNA or replacement of mouse genes with their human orthologues (lssDNA) [60, 61].

While the most commonly used model organism in biomedicine is mouse, CRISPR/Cas9 technology has also shown high efficiency in other laboratory animals such as rat, pigs and non-human primates [62–64]. These applications revealed the high potential of the CRISPR/Cas9 technology that allows to model diseases in animals that more comprehensively reflect human pathogenesis. However, some of animal models developed are far from being perfect and failed in translation to human pathophysiology, that negatively affects the development of innovative treatments. Nevertheless, the creating of transgenic animal models of human disorders have become a powerful approach for the discovery of new targets for therapeutic drugs.

Applications of CRISPR/Cas9 technology in biomedicine

In the past decades, gene therapy strategies have become important tools in the prevention and treatment of human diseases. It has been currently estimated that over 10000 human disorders are triggered by single mutated genes [65]. Due to successful development of genetic mouse model of the disorder, the first genetic drug Nusinersen™ to treat spinal muscular atrophy (SMA) was developed and approved by the U.S. Food and Drug Administration (FDA) in 2016, and another drug AVXS-101 is on a way for the first clinical trial in humans [66]. It is well known that SMA is neurodegenerative disease that caused by deletion in *SMN* gene, resulting in the deficiency in survival motor neuron (SMN) protein. Both drugs can be classified as targeting gene therapy products: Nusinersen™ is designed to bind a splicing silencer region on gene bringing a transcription of full-length SMN mRNA, and AVXS-101 is designed as a vector that carries DNA-encoding fully functional human target *SMN* gene [66, 67]. At present, FDA has approved 16 gene therapy drugs. However, these drugs are not involving direct gene editing, as in case with CRISPR/Cas technology. The first *ex vivo* CRISPR/spCas9 genome editing drug to treat the blood disorder β-thalassemia was created in 2014 and has been already submitted for a Phase I/II clinical trials in Europe [68]. The first *in vivo* CRISPR/spCas9 genome editing drug to treat Leber congenital amaurosis (LCA) were developed by the Allergan team in USA in 2017 and in 2019 was accepted for clinical trials by FDA [69, 70]. LCA10 is a severe retinal dystrophy caused by mutations in the *CEP290* gene. The CRISPR/Cas technology is also successfully used in the rapidly growing field of cancer treatment with patient-specific T cells that carry a Chimeric Antigen Receptor (CAR) on the cell membrane. The essence of CAR-T cell therapy is the genetically modification of T cells extracted from cancer patients (orthologous) or from healthy donors (allogeneic), followed by the reinfusion them into a cancer patient to find and kill cancerous cells. CARs 8 are artificial recombinant receptors designed to identify a specific antigen on tumor cells. CAR sequences are usually transduced into T cells using lentiviral viral vectors or are knocked in using the CRISPR/Cas system. In order to increase functionality CAR-T cells, for example to make them resistant to checkpoint inhibitors, or monoclonal antibody treatment, or patient's immune reaction, specific genes are removed from the surface of CAR-T cells by gene editing systems such as CRISPR/Cas [66–68]. Currently, several pharmaceutic companies have developing genome editing drugs. Some of them that are approved by FDA for clinical trials are listed in Table 2. It should be noted that clinical trials related to CRISPR/Cas technology are particularly actively conducted in China. Currently ten recruiting trials in China are listed on the US National Library of Medicine database - https://clinicaltrials.gov. Furthermore, CRISPR/Cas9 technology has been widely and successfully applied for various other biomedical applications in a number of fields ranging from therapy

Table 2. Selected genome editing programs approved by US FDA for clinical trials

Developer /therapy	Disease	The way of delivery	Description	NCT ID	Status
Servier Group company / UCART19	B-cell acute lymphoblastic leukemia	Intravenous infusion of TALEN gene-edited allogeneic CD19-targeted CAR-modified T cells.	Creating a knock out of the T-cell receptor (TCR) alpha chain (TRAC) and CD52 genes from the CD19-targeted CAR-T cells that can specifically recognize and bind to CD19-expressing tumor cells. Deletion of the CD52 gene makes the CAR19-T cells resistant to the monoclonal antibody alemtuzumab. The goal of TRAC gene deletion is to avoid immune reaction.	NCT02808442	Phase 1
Sangamo Therapeutics / SB-913	Mucopolysaccharidosis II (MPS II)	Intravenous infusion of AAV6 encoding ZFN genome editing therapy	Insertion the iduronate 2-sulfatase (IDS) gene under the control of the highly expressed albumin locus in liver cells to provide lifelong therapeutic production of the IDS enzyme.	NCT03041324	Phase 1/2
Vertex Pharmaceuticals Incorporated CRISPR Therapeutics / CTX-001	β-thalassemia, Sickle cell disease	Intravenous infusion of CRISPR/spCas9 gene-edited autologous hematopoietic CD34^{+}stem cells	The BCL11A protein is required to turn off production of fetal γ-globin in red cells. Knock out of BCL11A results in an increase production of fetal γ-globin instead of mutated beta-globin chains	NCT03745287	Phase 1/2
Allergan Editas Medicine, Inc. /AGN151587/EDIT-101	Leber congenital amaurosis type 10	Subretinal injection of AAV5 vector encoding CRISPR–saCas9 gene editing therapy	Repairing splicing defect in the CEP290 gene caused by the IVS26 mutation by specifically removing the intronic sequences flanking the mutation.	NCT03872479	Phase 1/2
CRISPR Therapeutics AG /CTX120	Multiple Myeloma	Intravenous infusion of CRISPR/spCas9 gene-edited allogeneic BCMA-targeted CAR-T-cells	Double knock out of the beta-2-microglobulin (B2M) and TRAC genes in allogeneic BCMA-targeted CAR-T-cells to eliminate their potential immunogenicity. B-cell maturation antigen (BCMA)- is a cell-surface receptor that is expressed in mature B cells, plasma cells and its overexpression and activation are associated with multiple myeloma.	NCT04244656	Phase 1
CRISPR Therapeutics AG / CTX110	B-cell Malignancy Non-Hodgkin Lymphoma B-cell Lymphoma	Intravenous infusion of CRISPR/spCas9 gene-edited allogeneic CD19-targeted CAR-T cells	The TCR deleted allogeneic CD19-targeted CAR-T cells can specifically identify and bind to CD19-expressing tumor cells and minimize the risk of immune reaction.	NCT04035434	Phase 1/2

and correction of heritable mutations in human embryos to modifying the pig genes for organs transplantation (Fig. 9) [71, 72]. Some of the most recent applications are listed in Table 3. Multiple studies are ongoing to improve gene therapy of a wide variety of single-gene disorders such as hemophilia, cystic fibrosis and other. In addition, there are many other applications that are being developed by using CRISPR/Cas9 technology. For example, using dCas9 as a transcriptional control has shown a potential application as a tool for modulation of aberrant DNA methylation that may be involved in a number of diseases including cancer [81].

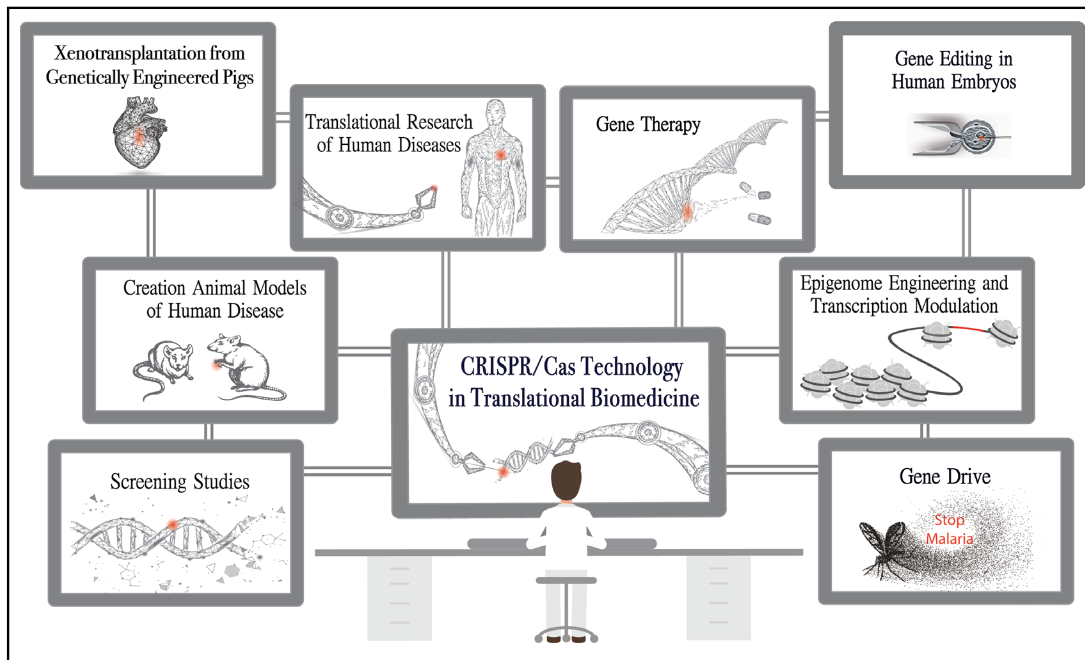

Fig. 9. Applications of CRISPR/Cas9 technology (please see details in the text).Fig. 8. Some of new approaches to generate genetically modified mice. A. FLP/FRT or Cre/LoxP site-directed recombination systems have been used to generate conditional knockout mutations. B. Direct transplantation of rAAV particles into the oviduct of pregnant females to generate knockout mice.Fig. 9. Applications of CRISPR/Cas9 technology.

Challenges

While it is expected that CRISPR technology could be safe and effective treatment approach, it is still in the beginning of the development as a therapy and there are many challenges and outstanding questions remain. For example, to cleave intron and restore the expression of wild-type *CEP290 gene* for LCA treatment, two sgRNA were used in order to activate NNEJ repair pathway. At present, gene therapy strategies that are primarily aimed at correcting the disease-causing mutations are mostly based on HR events. However, the efficiency of HR is low and taking place just during the late S/G2 phase of the cell cycle. Another challenge is a delivery tool, because a native size of Cas9 is too large to be packed in commonly used AAV vectors. Therefore, many investigators focused on discovering of smaller Cas proteins or finding out another delivery vehicles, such as nanoparticles carrying CRISPR/Cas9 constructs [82]. One more safety concern regarding clinical application of gene editing tools is unwanted off-target effects at genome regions that are highly identical to the sequence of interest. It is inspiring scientists to make improvements by optimizing sgRNA, modifying conformation of Cas9 or using anti-Cas9 inhibitors in order to limit the time of its action in the nucleus [83, 84]. It is necessary to emphasize also the existence of the ethical concern of this technology, because of the risks of inherited unpredictable off-target genetic mutations that can be worse than the therapeutic effect. While in February 2016 British scientists were given the permission to work with human embryos for research, it is clear that it is still premature to use this technology for clinical gene therapy involving embryos [85]. Before applying this technology for treatment of inherited disorders, it should be much better studied and tested, otherwise uncontrolled use of this approach may negatively affect the next generations.

Table 3. The examples of recent applications of CRISPR/Cas9 system in therapeutic research projects

Year	Country	Disorder	Description
2017	USA	Huntington's disease is a fatal neurodegenerative disorder caused by CAG repeat expansion in the HTT gene.	CRISPR/Cas9 construct development for allele-specific genome-editing of mutant huntingtin gene (HTT) to reduce the expression of mutant HTT alleles in human fibroblasts with Huntington disease and mouse model of Huntington disease [73].
2017	Switzerland	Huntington's disease	Development of KamiCas9 self-inactivating editing system used for inactivating mutant HTT in mouse models of Huntington's disease [74].
2017	USA	The mutations in MYBPC3 gene cause the heart muscle to thicken resulting in hypertrophic cardiomyopathy.	Modification of the MYBPC3 gene in embryos using CRISPR/Cas9 system [75].
2018	Japan	Human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS)	Effective inhibition of HIV-1 replication in persistently infected $CD4^+$ T-cell lines as well as latently infected T cells with the help of tat and rev-targeting CRISPR/Cas9 lentiviral constructs [76].
2018	China, Russia	HIV/AIDS A genetic mutation known as CCR5-delta 32 is responsible for the HIV resistance. It is known that HIV requires co-receptor CCR5 on the surface of immune cells to get inside the cells.	A novel approach by developing HIV resistance via induction of the CCR5delta32 mutation. Such mutation was successfully performed in human embryos by Russian scientists [77]. Furthermore, a Chinese scientist in 2018 alerted the world by the claim of first humans that were born from a CRISPR/CAS9 gene-edited embryos.
2019	USA	Duchenne muscular dystrophy is X-linked recessive disorders characterized by lethal degeneration of cardiac and skeletal muscles caused by mutations in the DMD gene, encoding the dystrophin protein. The most common mutation is deletion of exon 44 (ΔEx44) in the DMD gene resulting in an out-of-frame stop mutation of dystrophin.	CRISPR-Cas9 gene editing construct development for removing the splice acceptor or donor sequence of the out-of-frame exon to restore the protein reading frame in cardiomyocytes with Duchenne muscular dystrophy and in the mouse model with the same mutation [78].
2019	Spanish	Hutchinson–Gilford progeria syndrome is a rare disease characterized by aging-like appearance in childhood, caused by point mutation in the LMNA gene, which activates an undesirable splice site in exon 11 and as a result expression of progerin.	Development of CRISPR-Cas9 gene editing construct to disrupt the last part of the LMNA gene in order to block the translation and accumulation of progerin in cells and mouse model [79].
2019	USA	Hemophilia A is a X-linked monogenic disease caused by mutations in coagulation factor VIII encoded by the f8 gene. Hemophilia patients are characterized by uncontrolled spontaneous. The disease usually affects males.	Development of CRISPR-Cas9-based in vivo gene editing construct to knock in a modified human B domain deleted-F8 (BDD-F8) at the albumin (Alb) locus in liver cells of hemophilic mice [80].

Conclusion

In conclusion, CRISPR-Cas9 technology, that was first described as a programming editing tool in mammals in 2012, just in few years has revolutionized investigations in various fields in molecular biology and translational biomedicine. Manipulation of the laboratory animal genomes by CRISPR-Cas9 method have already contributed to the understanding of many functions of genes and has become a commonly used tool for modeling human disorders, thereby leading to multiple advances in disease diagnosis, the implementation of targeted therapeutics and personalized medicine strategies. There are still certain challenges that need to be overcome for the safe and effective use of CRISPR/Cas technology in clinical gene therapy applications. Nevertheless, CRISPR/Cas-mediated genome editing has already demonstrated utility in multiple preclinical and clinical investigations focused on pathophysiological mechanisms of various human genetic diseases and has an enormous potential as an effective tool to make genomic engineering manipulations in clinical practice more routine.

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Statement of Ethics
The authors have no ethical conflicts to disclose.

Author Contributions
All authors contributed to the drafting and critical revision of this manuscript.

Disclosure Statement

The authors have no conflicts of interest to declare.

References

1 Xie H, Tang L, He X, Liu X, Zhou C, Liu J, Ge X, Li J, Liu C, Zhao J, Qu J, Song Z, Gu F: SaCas9 Requires 5′-NNGRRT-3′ PAM for Sufficient Cleavage and Possesses Higher Cleavage Activity than SpCas9 or FnCpf1 in Human Cells. Biotechnol J 2018;13:1700561.
2 van Soolingen D, de Haas PE, Hermans PW, Groenen PM, van Embden JD: Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of Mycobacterium tuberculosis. J Clin Microbiol 1993;31:1987-1995.
3 Mojica FJ, Ferrer C, Juez G, Rodríguez-Valera F: Long stretches of short tandem repeats are present in the largest replicons of the Archaea Haloferax mediterranei and Haloferax volcanii and could be involved in replicon partitioning. Mol Microbiol 1995;17:85-93.
4 Mojica FJ, Díez-Villaseñor C, Soria E, Juez G: Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. Mol Microbiol 2000;36:244-246.
5 Bolotin A, Quinquis B, Sorokin A, Ehrlich SD: Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology 2005;151:2551-2561.
6 Mojica FJM, Díez-Villaseñor C, García-Martínez Js, Soria E: Intervening Sequences of Regularly Spaced Prokaryotic Repeats Derive from Foreign Genetic Elements. J Mol Evol 2005;60:174-182.
7 Pourcel C, Salvignol G, Vergnaud G: CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. Microbiology 2005;151:653-663.
8 Makarova KS, Grishin NV, Shabalina SA, Wolf YI, Koonin EV: A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. Biol Direct 2006;1:7.
9 Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E: A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science 2012;337:816-821.
10 Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F: Multiplex Genome Engineering Using CRISPR/Cas Systems. Science 2013;339:819-823.
11 Koonin EV, Makarova KS, Zhang F: Diversity, classification and evolution of CRISPR-Cas systems. Curr Opin Microbiol 2017;37:67-78.
12 Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DBT, Shmakov S, Makarova KS, Semenova E, Minakhin L, Severinov K, Regev A, Lander ES, Koonin EV, Zhang F: C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science 2016;353:aaf5573.
13 Abudayyeh OO, Gootenberg JS, Essletzbichler P, Han S, Joung J, Belanto JJ, Verdine V, Cox DBT, Kellner MJ, Regev A, Lander ES, Voytas DF, Ting AY, Zhang F: RNA targeting with CRISPR–Cas13. Nature 2017;550:280-284.
14 Anders C, Niewoehner O, Duerst A, Jinek M: Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. Nature 2014;513:569-573.

15 Jinek M, Jiang F, Taylor DW, Sternberg SH, Kaya E, Ma E, Anders C, Hauer M, Zhou K, Lin S, Kaplan M, Iavarone AT, Charpentier E, Nogales E, Doudna JA: Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. Science 2014;343:1247997.
16 Nishimasu H, Ran FA, Hsu Patrick D, Konermann S, Shehata Soraya I, Dohmae N, Ishitani R, Zhang F, Nureki O: Crystal Structure of Cas9 in Complex with Guide RNA and Target DNA. Cell 2014;156:935-949.
17 Jiang F, Zhou K, Ma L, Gressel S, Doudna JA: A Cas9-guide RNA complex preorganized for target DNA recognition. Science 2015;348:1477-1481.
18 Anders C, Bargsten K, Jinek M: Structural Plasticity of PAM Recognition by Engineered Variants of the RNA-Guided Endonuclease Cas9. Mol Cell 2016;61:895-902.
19 Huai C, Li G, Yao R, Zhang Y, Cao M, Kong L, Jia C, Yuan H, Chen H, Lu D, Huang Q: Structural insights into DNA cleavage activation of CRISPR-Cas9 system. Nat Commun 2017;8:1375.
20 Cymerman IA, Obarska A, Skowronek KJ, Lubys A, Bujnicki JM: Identification of a new subfamily of HNH nucleases and experimental characterization of a representative member, HphI restriction endonuclease. Proteins 2006;65:867-876.
21 Górecka KM, Komorowska W, Nowotny M: Crystal structure of RuvC resolvase in complex with Holliday junction substrate. Nucleic Acids Res 2013;41:9945-9955.
22 Labuhn M, Adams FF, Ng M, Knoess S, Schambach A, Charpentier EM, Schwarzer A, Mateo JL, Klusmann JH, Heckl D: Refined sgRNA efficacy prediction improves large- and small-scale CRISPR–Cas9 applications. Nucleic Acids Res 2018;46:1375-1385.
23 Ran FA, Hsu Patrick D, Lin CY, Gootenberg Jonathan S, Konermann S, Trevino AE, Scott David A, Inoue A, Matoba S, Zhang Y, Zhang F: Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity. Cell 2013;154:1380-1389.
24 Xu H, Xiao T, Chen CH, Li W, Meyer CA, Wu Q, Wu D, Cong L, Zhang F, Liu JS, Brown M, Liu XS: Sequence determinants of improved CRISPR sgRNA design. Genome Res 2015;25:1147-1157.
25 Chatterjee P, Jakimo N, Jacobson JM: Minimal PAM specificity of a highly similar SpCas9 ortholog. Sci Adv 2018;4:eaau0766.
26 Hirano H, Gootenberg Jonathan S, Horii T, Abudayyeh Omar O, Kimura M, Hsu Patrick D, Nakane T, Ishitani R, Hatada I, Zhang F, Nishimasu H, Nureki O: Structure and Engineering of Francisella novicida Cas9. Cell 2016;164:950-961.
27 Garneau JE, Dupuis M-È, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadán AH, Moineau S: The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature 2010;468:67-71.
28 Magadán AH, Dupuis M-È, Villion M, Moineau S: Cleavage of Phage DNA by the Streptococcus thermophilus CRISPR3-Cas System. PLoS One 2012;7:e40913.
29 Lee CM, Cradick TJ, Bao G: The Neisseria meningitidis CRISPR-Cas9 System Enables Specific Genome Editing in Mammalian Cells. Mol Ther 2016;24:645-654.
30 Edraki A, Mir A, Ibraheim R, Gainetdinov I, Yoon Y, Song C-Q, Cao Y, Gallant J, Xue W, Rivera-Pérez JA, Sontheimer EJ: A Compact, High-Accuracy Cas9 with a Dinucleotide PAM for In Vivo Genome Editing. Mol Cell 2019;73:714-726.e714.
31 Briner AE, Barrangou R: Lactobacillus buchneri Genotyping on the Basis of Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) Locus Diversity. Appl Environ Microbiol 2014;80:994-1001.
32 Yang W: An equivalent metal ion in one- and two-metal-ion catalysis. Nat Struct Mol Biol 2008;15:1228-1231.
33 San Filippo J, Sung P, Klein H: Mechanism of Eukaryotic Homologous Recombination. Annu Rev Biochem 2008;77:229-257.
34 Krejci L, Altmannova V, Spirek M, Zhao X: Homologous recombination and its regulation. Nucleic Acids Res 2012;40:5795-5818.
35 McVey M, Khodaverdian VY, Meyer D, Cerqueira PG, Heyer WD: Eukaryotic DNA Polymerases in Homologous Recombination. Annu Rev Genet 2016;50:393-421.
36 Boel A, De Saffel H, Steyaert W, Callewaert B, De Paepe A, Coucke PJ, Willaert A: CRISPR/Cas9-mediated homology-directed repair by ssODNs in zebrafish induces complex mutational patterns resulting from genomic integration of repair-template fragments. Dis Model Mech 2018;11:pii:dmm035352.
37 Cubbon A, Ivancic-Bace I, Bolt Edward L: CRISPR-Cas immunity, DNA repair and genome stability. Biosci Rep 2018;38:pii:BSR20180457.
38 Lieber MR: The Mechanism of Double-Strand DNA Break Repair by the Nonhomologous DNA End-Joining Pathway. Annu Rev Biochem 2010;79:181-211.

39 Nick McElhinny SA, Havener JM, Garcia-Diaz M, Juárez R, Bebenek K, Kee BL, Blanco L, Kunkel TA, Ramsden DA: A Gradient of Template Dependence Defines Distinct Biological Roles for Family X Polymerases in Nonhomologous End Joining. Mol Cell 2005;19:357-366.
40 Chang HHY, Pannunzio NR, Adachi N, Lieber MR: Non-homologous DNA end joining and alternative pathways to double-strand break repair. Nat Rev Mol Cell Biol 2017;18:495-506.
41 Truong LN, Li Y, Shi LZ, Hwang PY-H, He J, Wang H, Razavian N, Berns MW, Wu X: Microhomology-mediated End Joining and Homologous Recombination share the initial end resection step to repair DNA double-strand breaks in mammalian cells. Proc Natl Acad Sci U S A 2013;110:7720-7725.
42 Mateos-Gomez PA, Gong F, Nair N, Miller KM, Lazzerini-Denchi E, Sfeir A: Mammalian polymerase θ promotes alternative NHEJ and suppresses recombination. Nature 2015;518:254-257.
43 Sfeir A, Symington LS: Microhomology-Mediated End Joining: A Back-up Survival Mechanism or Dedicated Pathway? Trends Biochem Sci 2015;40:701-714.
44 Masani S, Han L, Meek K, Yu K: Redundant function of DNA ligase 1 and 3 in alternative end-joining during immunoglobulin class switch recombination. Proc Natl Acad Sci U S A 2016;113:1261-1266.
45 Taleei R, Nikjoo H: Biochemical DSB-repair model for mammalian cells in G1 and early S phases of the cell cycle. Mutat Res 2013;756:206-212.
46 Kakarougkas A, Jeggo PA: DNA DSB repair pathway choice: an orchestrated handover mechanism. Br J Radiol 2014;87:20130685.
47 Brandsma I, Gent DC: Pathway choice in DNA double strand break repair: observations of a balancing act. Genome Integr 2012;3:9.
48 Lin S, Staahl BT, Alla RK, Doudna JA: Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. eLife 2014;3:e04766.
49 Liang X, Potter J, Kumar S, Ravinder N, Chesnut JD: Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA. J Biotechnol 2017;241:136-146.
50 Mao Z, Bozzella M, Seluanov A, Gorbunova V: Comparison of nonhomologous end joining and homologous recombination in human cells. DNA Repair 2008;7:1765-1771.
51 Zuo E, Cai YJ, Li K, Wei Y, Wang BA, Sun Y, Liu Z, Liu J, Hu X, Wei W, Huo X, Shi L, Tang C, Liang D, Wang Y, Nie YH, Zhang CC, Yao X, Wang X, et al.: One-step generation of complete gene knockout mice and monkeys by CRISPR/Cas9-mediated gene editing with multiple sgRNAs. Cell Res 2017;27:933-945.
52 Miyasaka Y, Uno Y, Yoshimi K, Kunihiro Y, Yoshimura T, Tanaka T, Ishikubo H, Hiraoka Y, Takemoto N, Tanaka T, Ooguchi Y, Skehel P, Aida T, Takeda J, Mashimo T: CLICK: one-step generation of conditional knockout mice. BMC Genomics 2018;19:318.
53 Yoon Y, Wang D, Tai PWL, Riley J, Gao G, Rivera-Pérez JA: Streamlined ex vivo and in vivo genome editing in mouse embryos using recombinant adeno-associated viruses. Nat Commun 2018;9:412.
54 Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F: DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol 2013;31:827-832.
55 Karousis ED, Nasif S, Mühlemann O: Nonsense-mediated mRNA decay: novel mechanistic insights and biological impact: Nonsense-mediated mRNA decay. Wiley Interdiscip Rev RNA 2016;7:661-682.
56 Zhang H, McCarty N: CRISPR-Cas9 technology and its application in haematological disorders. Br J Haematol 2016;175:208-225.
57 Yang F, Liu C, Chen D, Tu M, Xie H, Sun H, Ge X, Tang L, Li J, Zheng J, Song Z, Qu J, Gu F: CRISPR/Cas9-loxP -Mediated Gene Editing as a Novel Site-Specific Genetic Manipulation Tool. Mol Ther Nucleic Acids 2017;7:378-386.
58 Horii T, Morita S, Kimura M, Terawaki N, Shibutani M, Hatada I: Efficient generation of conditional knockout mice via sequential introduction of lox sites. Sci Rep 2017;7:7891.
59 Nagy A: Cre recombinase: the universal reagent for genome tailoring. Genesis 2000;26:99-109.
60 Quadros RM, Miura H, Harms DW, Akatsuka H, Sato T, Aida T, Redder R, Richardson GP, Inagaki Y, Sakai D, Buckley SM, Seshacharyulu P, Batra SK, Behlke MA, Zeiner SA, Jacobi AM, Izu Y, Thoreson WB, Urness LD, Mansour SL, et al.: Easi-CRISPR: a robust method for one-step generation of mice carrying conditional and insertion alleles using long ssDNA donors and CRISPR ribonucleoproteins. Genome Biol 2017;18:92.
61 He X, Li YX, Feng B: New Turns for High Efficiency Knock-In of Large DNA in Human Pluripotent Stem Cells. Stem Cells Int 2018;2018:1-7.
62 Yang H, Wu Z: Genome Editing of Pigs for Agriculture and Biomedicine. Front Genet 2018;9:360.

63 Sato K, Sasaki E: Genetic engineering in nonhuman primates for human disease modeling. J Hum Genet 2018;63:125-131.
64 Remy S, Chenouard V, Tesson L, Usal C, Ménoret S, Brusselle L, Heslan J-M, Nguyen TH, Bellien J, Merot J, De Cian A, Giovannangeli C, Concordet J-P, Anegon I: Generation of gene-edited rats by delivery of CRISPR/Cas9 protein and donor DNA into intact zygotes using electroporation. Sci Rep 2017;7:16554.
65 Driss A, Asare KO, Hibbert JM, Gee BE, Adamkiewicz TV, Stiles JK: Sickle Cell Disease in the Post Genomic Era: A Monogenic Disease with a Polygenic Phenotype. Genomics Insights 2009;2009:23-48.
66 Rao VK, Kapp D, Schroth M: Gene Therapy for Spinal Muscular Atrophy: An Emerging Treatment Option for a Devastating Disease. J Manag Care Spec Pharm 2018;24:S3-S16.
67 Foust KD, Wang X, McGovern VL, Braun L, Bevan AK, Haidet AM, Le TT, Morales PR, Rich MM, Burghes AHM, Kaspar BK: Rescue of the spinal muscular atrophy phenotype in a mouse model by early postnatal delivery of SMN. Nat Biotechnol 2010;28:271-274.
68 Xie F, Ye L, Chang JC, Beyer AI, Wang J, Muench MO, Kan YW: Seamless gene correction of β-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac. Genome Res 2014;24:1526-1533.
69 Ruan G-X, Barry E, Yu D, Lukason M, Cheng SH, Scaria A: CRISPR/Cas9-Mediated Genome Editing as a Therapeutic Approach for Leber Congenital Amaurosis 10. Mol Ther 2017;25:331-341.
70 Sheridan C: Go-ahead for first in-body CRISPR medicine testing. Nat Biotechnol 2018; DOI:10.1038/d41587-018-00003-2.
71 Naeimi Kararoudi M, Hejazi SS, Elmas E, Hellström M, Naeimi Kararoudi M, Padma AM, Lee D, Dolatshad H: Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 Gene Editing Technique in Xenotransplantation. Front Immunol 2018;9:1711.
72 Schenkwein D, Ylä-Herttuala S: Gene Editing of Human Embryos with CRISPR/Cas9: Great Promise Coupled with Important Caveats. Mol Ther 2018;26:659-660.
73 Monteys AM, Ebanks SA, Keiser MS, Davidson BL: CRISPR/Cas9 Editing of the Mutant Huntingtin Allele In Vitro and In Vivo. Mol Ther 2017;25:12-23.
74 Merienne N, Vachey G, de Longprez L, Meunier C, Zimmer V, Perriard G, Canales M, Mathias A, Herrgott L, Beltraminelli T, Maulet A, Dequesne T, Pythoud C, Rey M, Pellerin L, Brouillet E, Perrier AL, du Pasquier R, Déglon N: The Self-Inactivating KamiCas9 System for the Editing of CNS Disease Genes. Cell Rep 2017;20:2980-2991.
75 Ledford H: CRISPR fixes disease gene in viable human embryos. Nature 2017;548:13-14.
76 Ophinni Y, Inoue M, Kotaki T, Kameoka M: CRISPR/Cas9 system targeting regulatory genes of HIV-1 inhibits viral replication in infected T-cell cultures. Sci Rep 2018;8:7784.
77 Kodyleva TA, Kirillova AO, Tyschik EA, Makarov VV, Khromov AV, Guschin VA, Abubakirov AN, Rebrikov DV, Sukhikh GT: The efficacy of CRISPR-Cas9-mediated induction of the CCR5delta32 mutation in the human embryo. Bulletin of Russian State Medical University 2018:70-74.
78 Min YL, Li H, Rodriguez-Caycedo C, Mireault AA, Huang J, Shelton JM, McAnally JR, Amoasii L, Mammen PPA, Bassel-Duby R, Olson EN: CRISPR-Cas9 corrects Duchenne muscular dystrophy exon 44 deletion mutations in mice and human cells. Sci Adv 2019;5:eaav4324.
79 Santiago-Fernández O, Osorio FG, Quesada V, Rodríguez F, Basso S, Maeso D, Rolas L, Barkaway A, Nourshargh S, Folgueras AR, Freije JMP, López-Otín C: Development of a CRISPR/Cas9-based therapy for Hutchinson–Gilford progeria syndrome. Nat Med 2019;25:423-426.
80 Chen H, Shi M, Gilam A, Zheng Q, Zhang Y, Afrikanova I, Li J, Gluzman Z, Jiang R, Kong L-J, Chen-Tsai RY: Hemophilia A ameliorated in mice by CRISPR-based in vivo genome editing of human Factor VIII. Sci Rep 2019;9:16838.
81 Lei Y, Zhang X, Su J, Jeong M, Gundry MC, Huang Y-H, Zhou Y, Li W, Goodell MA: Targeted DNA methylation in vivo using an engineered dCas9-MQ1 fusion protein. Nat Commun 2017;8:16026.
82 Biagioni A, Laurenzana A, Margheri F, Chillà A, Fibbi G, Del Rosso M: Delivery systems of CRISPR/Cas9-based cancer gene therapy. J Biol Eng 2018;12:33.
83 Sontheimer EJ, Davidson AR: Inhibition of CRISPR-Cas systems by mobile genetic elements. Curr Opin Microbiol 2017;37:120-127.
84 Hu JH, Miller SM, Geurts MH, Tang W, Chen L, Sun N, Zeina CM, Gao X, Rees HA, Lin Z, Liu DR: Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. Nature 2018;556:57-63.
85 Rodriguez E: Ethical Issues in Genome Editing using Crispr/Cas9 System. J Clin Res Bioeth 2016;7:1-4.