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.

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...
[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, Class 2 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 resolvasse 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
(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...
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**

<table>
<thead>
<tr>
<th>Cas9 ortholog</th>
<th>PAM</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>5'-NGG-3' (N-any)</td>
<td>[9, 17]</td>
</tr>
<tr>
<td>(SpCas9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Francisella novicida</em></td>
<td>5'-YG-3' (Y- pyrimidines)</td>
<td>[26]</td>
</tr>
<tr>
<td>(FnCas9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>5'-NNGRRT-3' (R- purines)</td>
<td>[1]</td>
</tr>
<tr>
<td>(SaCas9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus thermophiles</em></td>
<td>5'-NNAGAAW-3' (W- A or T)</td>
<td>[27]</td>
</tr>
<tr>
<td>(St1Cas9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus thermophiles</em></td>
<td>5'-NGGNG</td>
<td>[28]</td>
</tr>
<tr>
<td>(St3Cas9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>5'-NNNGATT-3'</td>
<td>[29]</td>
</tr>
<tr>
<td>(NmCas9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>5'-NNNNCC-3'</td>
<td>[30]</td>
</tr>
<tr>
<td>(Nme2Cas9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus buchneri</em></td>
<td>5'-AAAAA-3'</td>
<td>[31]</td>
</tr>
<tr>
<td>(LbCas9)</td>
<td></td>
<td></td>
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</table>
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.

**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 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 G2 (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 overhanging 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

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**Model for HR repair**

![Schematic illustration of HR repair model in mammalian cells](image)

*Fig. 5. Schematic illustration of HR 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 

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**Fig. 6.** Schematic illustration of NHEJ repair model in mammalian cells (please see details in the text).
crucial elements of drug development by providing \textit{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 \textit{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 have 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
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 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
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].
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 NHEJ 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

<table>
<thead>
<tr>
<th>Year</th>
<th>Country</th>
<th>Disorder</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2017</td>
<td>USA</td>
<td>Huntington’s disease is a fatal neurodegenerative disorder caused by CAG repeat expansion in the HTT gene.</td>
<td>CRISPR/Cas9 construct development for allele-specific genome-editing of mutant huntington gene (HTT) to reduce the expression of mutant HTT alleles in human fibroblasts with Huntington disease and mouse model of Huntington disease [73].</td>
</tr>
<tr>
<td>2017</td>
<td>Switzerland</td>
<td>Myocardial infarction resulting in hypertrophic cardiomyopathy.</td>
<td>Development of KamnCas9 self-inactivating editing system used for inactivating mutant HTT in mouse models of Huntington’s disease [74].</td>
</tr>
<tr>
<td>2017</td>
<td>USA</td>
<td>The mutations in MYBPC3 gene cause the heart muscle to thicken resulting in hypertrophic cardiomyopathy.</td>
<td>Modification of the MYBPC3 gene in embryos using CRISPR/Cas9 system [75].</td>
</tr>
<tr>
<td>2018</td>
<td>Japan</td>
<td>Human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS)</td>
<td>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].</td>
</tr>
<tr>
<td>2018</td>
<td>China, Russia</td>
<td>HIV/AIDS</td>
<td>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.</td>
</tr>
<tr>
<td>2019</td>
<td>USA</td>
<td>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.</td>
<td>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].</td>
</tr>
<tr>
<td>2019</td>
<td>Spain</td>
<td>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.</td>
<td>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].</td>
</tr>
<tr>
<td>2019</td>
<td>USA</td>
<td>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.</td>
<td>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 hemophiliac mice [80].</td>
</tr>
</tbody>
</table>

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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The authors have no ethical conflicts to disclose.

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