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The Current State-of-the-Art in Therapeutic Genome Editing and the Future

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Abstract

Genome editing using designer site-specific nucleases is a burgeoning field in which genomes of target cells/organisms are now being manipulated to create/correct mutations or transcriptionally manipulate gene expression. The field began over a decade ago with zinc finger nucleases, which were soon followed by designer homing endonucleases/mega nucleases, transcription activator-like effector nucleases, and more recently, CRISPR/Cas9. Each platform has its own strengths and weaknesses but they all allow editing of the genome in cells and organisms to either study biology/function of genes or for a therapeutic effect. This review will briefly describe the various gene editing platforms and then focus in on the CRISPR/Cas9 system.

Keywords: Mega nucleases; CRISPR/Cas9; microRNA

Introduction

Over the past two decades, significant improvements have been made in virus-based gene transfer approaches for the treatment of disease by restoration of a missing gene to cells or tissues or to knockdown genes for therapeutic effect or study disease biology by RNA interference (RNAi). Despite their initial successes in the treatment of monogenic recessive disorders [1,2] and the enhanced safety mechanisms built into the virus-based gene replacement therapy approaches, these still have some limitations. First, the transgene and its regulatory elements need to be inserted into the vector, and often cargo carried is too large for the payload of the viral vectors. Second, the random integration of the transgene cassette has a potential, albeit small, of unintentional vector-mediated insertional proto-oncogene activation or dysregulation of an endogenous gene [3-5]. For RNAi approaches, while these are extensively used to study biological processes and gene function, there are concerns regarding the specificity and safety of permanently inserting shRNA or shRNA embedded into microRNA in the genome for clinical translation [6]. Regardless, gene replacement therapy has been successfully translated to the clinic and is curing a majority of patients. However, the inserted genes are not under control of the endogenous gene regulation machinery, which would provide optimal level and regulation of expression. Genome editing with programmable nucleases such as mega nucleases [7], zinc finger nucleases (ZFNs) [8], transcription activator-like effector nucleases (TALENs) [9], and clustered regulatory interspaced short palindromic repeat (CRISPR) and CRIPSR-associated protein (CRISPR-Cas) [10] enables precise and efficient genome targeting, which can be used for gene disruption or homologous recombination, without the need for introducing permanent exogenous genetic material into the host genome. This article briefly describes the current state of the nuclease-based genome editing approaches, with a special focus on CRISPR-Cas technology.

Gene Editing by Reprogrammable Nucleases

Precise genome editing with reprogrammable nucleases such as mega nucleases, ZFNs, TALENs, and CRISPR-Cas involves the introduction of site-specific DNA double-strand break (DSBs) into the genome by these nucleases. Generally, DNA DSBs are repaired via either non-homologous end joining (NHEJ) or homologous recombination (HR), with NHEJ as the default repair pathway that occurs following a DSB by these nucleases, in absence of a donor DNA template [3]. In the presence of a DNA template, which has homology on the 5' and 3' ends around the region of the DSB, homology directed repair can occur. Furthermore, micro homology-mediated end joining (MMEJ) is an alternate end joining repair pathway that also repairs DSBs through the use of micro homologous sequences, resulting in deletions of genetic material [11]. NHEJ, the predominant DSBs repair pathway in mammalian cells, mediates DSB repair by direct rejoining of the two DSB ends in an error-prone manner, leading to the introduction of small insertions/deletions (indels). Indels can disrupt the reading frame of the coding region of a gene, leading to the production of truncated proteins or mRNA degradation by nonsense-mediated decay [3,12]. Hence, NHEJ can be used to create gene knockouts. Unlike NHEJ, error-free HR-mediated DSB repair requires the presence of a homologous DNA template. By providing single- or double-stranded exogenous DNA templates, precise, targeted changes to the genome can be done [3].

Several factors including cell type and cell-cycle status, the size of the deletion/insertion, and the length of the homology arm of the donor DNA can affect the efficiency of genome editing by HR [3]. For example, unlike NHEJ mediated repair, which can occur regardless of the cycling status of the cell, HR is mainly active during the S/G2 phase, limiting precise genome modifications to only mitotic cells [3,13,14]. Moreover, it has been reported that single-stranded DNA provides superior HR rates compared to double-stranded DNA [15]. Furthermore, longer homology arms have been shown to be associated with a higher HR rate, while an inverse relationship has been shown between HR rates and the length of the HR-mediated insertions [16].

Gene Editing Approaches

Mega nucleases

Homing endonucleases are a large family of DNA endonucleases derived from phages, bacteria, archaebacteria and eukaryotes. Despite their small size (<40 K Da), homing endonucleases have extended (12-

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45 bp) DNA recognition sites [17]. Homing endonucleases are usually embedded within self-splicing elements (Group I intron, Group II intron, or intein), which avoids disruption of the target gene, while the activity of the endonuclease results in a highly efficient site-specific recombination event [7,18]. At least 5 families of homing endonucleases have been characterized, which vary highly in their conserved nuclease core motif, mechanism of catalysis, and host organism [7,19]. Of the 5 families, the enzymes from the LAGLIDADG homing endonuclease family, which are usually termed as mega nucleases, are the most widely used homing endonucleases for gene editing [7].

Mega nuclease-induced DSBs are repaired via HR [17], enabling precise genome editing. Mega nuclease-mediated gene editing, however, has its limitations. One of the major drawbacks of mega nucleases is the need for introduction of a known long cleavage site into the region of interest. In addition, because the DNA recognition and cleavage domains of mega nucleases are structurally associated, engineering mega nucleases, without disrupting their overall stability, has been challenging [17]. However, one major advantage in the use of mega nucleases is that, due to their small size, their delivery both *in vivo* and *ex vivo* is relatively easy.

Zinc finger nucleases (ZFNs)

ZFNs are chimeric synthetic endonucleases, with a 9-18 bp recognition site per ZFN, consisting of a zinc finger DNA-binding domain fused to the FokI DNA cleavage domain. Like mega nucleases, ZFNs can be manipulated to target a specific DNA sequence [3]. However, designing custom ZFNs that have high specificity to target sequences requires substantial protein engineering, which has been rate limiting, preventing their widespread use [20]. It was reported that ZFNs can have substantial off-target effects at undesired sites; however, heterodimerization has helped reduce these off-target effects [21]. The advantage of ZFNs, like meganucleases, is their small size, which facilitates their delivery both *in vivo* and *ex vivo* [3].

Several preclinical studies have reported successful application of ZFNs to genome editing in murine models of hemophilia B, when injected *in vivo* [22], and in severe combined immune deficiency (SCID) [23], and in HIV-infected mice with editing performed ex vivo [24,25]. For example, it has previously been shown that ZFN can efficiently mediate HR targeted gene insertion in mouse liver, and that the level of correction was sufficient to correct hemophilia B phenotype in mouse model of hemophilia B [22]. ZFN has also been successfully applied to genome editing in patient-derived induced pluripotent stem cells (iPSCs) [26-29]. More recently, ZFN that help disrupt CCR5 surface expression on T cells have completed early phase safety and efficacy testing in HIV infected humans [30].

Transcription activator-like effector nucleases (TALENs)

TALENs are chimeric restriction endonucleases, with a 14-20 bp recognition site per TALEN. TALENs are comprised of a DNA recognition domain and a DNA cleavage domain fused together. Similar to mega nucleases and ZFNs, TALENs can be engineered to target a specific DNA sequence. While designing TALENs is relatively simple, TALEN-coding sequences are quite long, and their generation involves rather complicated molecular cloning [3]. A disadvantage of the TALEN system is the large size of the TALENs which can impede *in vivo* delivery. TALEN-mediated NHEJ has been previously reported to restore the mutated dystrophin reading frame in cells from patients with duchenne muscular dystrophy (DMD) [31]. In addition, TALEN-mediated NHEJ has been used to inhibit viral replication in a mouse model of hepatitis B virus (HBV) replication established by hydrodynamic injection [32]. Furthermore, TALEN has been used for genome editing in patient-derived iPSCs including those from patients with X-linked chronic granulomatous [33], β-thalassemia [34] and sickle cell disease [35].

CRISPR-Cas

The CRISPR-Cas system exploits the mechanism used by bacteria to recognize and cleave re-infecting phage DNA. Unlike the mega nucleases, ZFNs and TALENs, in which a site-specific DNA-binding domain directs the nuclease to the desired target site, in CRISPR-Cas systems, specific DNA binding is achieved by a short RNA guide molecule [3]. The major advantage of the CRISPR-Cas system is that, unlike the above-mentioned systems, CRISPR-Cas can be readily engineered to target a new DNA sequence by changing a 20-nucleotide sequence within the guide RNA molecule which re-directs the Cas nuclease to a desired target site through Watson-Crick base paring [3,36].

Based on the components of Cas proteins, CRISPR-Cas systems can be broadly divided into two classes. Class I (types I, III and IV) CRISPR-Cas systems employ various Cas nucleases while Class II (types II and V) CRISPR-Cas systems use a large single-component Cas nuclease [37,38]. A CRISPR-Cas system is composed of the CRISPR RNA (crRNA) array consisting of Cas gene, non-coding RNAs and direct repeats interspaced by protospacers. Protospacers are flanked by sequences known as protospacer adjacent motifs (PAMs) within the DNA target. Among the CRISPR-Cas systems, the type II system is the best characterized and has been adopted for precise genome editing [36] A brief review of the CRISPR-Cas systems utilized for genome editing is presented below.

CRISPR-Cas9

The type II CRISPR system from Streptococcus pyogenes, consisting of the Cas9 nuclease, a crRNA array, and an auxiliary trans-activation crRNA (tracrRNA), has been adopted for gene editing. Herein, crRNA, which consists of a 20-nucleotide guide sequence and a short direct repeat, directs the Cas9 nuclease to a target DNA sequence followed by a G-rich PAM (5'-NGG) (Figure 1A). The Cas9 protein contains predicted RuvC-like and HNH endonuclease domains**.** HNH cleaves the DNA strand complementary to the guide RNA while RuvC mediates cleavage of the noncomplementary strand. Of note, to simplify the CRIPSR/Cas system for gene editing, a chimeric single-guide RNA (sgRNA) has been generated by fusing crRNA and tracrRNA [36].

Despite its great promise, a number of limitations exist with the CRISPR-Cas9 system. One of the major limitations of the CRISPR-Cas9 system is the large 4.5 kb size of the Cas9 nuclease derived from streptococcus pyogenes (spCas9) which limits its application in therapeutic genome editing; for example, the size precludes its delivery via adeno-associated virus (AAV), one of the most promising viral vectors for gene therapy. To overcome this limitation, a smaller Cas9 from staphylococcus aureus (saCas9) has been recently described with cleavage efficiency comparable to that of spCas9 despite being slightly shorter (3.2 kb) [37]. Interestingly, saCas9 has shown higher specificity compared to spCas9, presumably due to the long 5′-NNGRRT PAM of saCas9 (Figure 1B). More recently, to overcome the size limitation of spCas9, a 'split spCas9' has been generated, where the coding sequences of the recognition (REC) lobe and the nuclease (NUC) lobe of spCas9 were distributed across two separate vectors. The separate lobes were later dimerized into a fully functional spCas9 enzyme using split inteins [39] or by the use of FKBP dimerization domain and spCas9 dimerized using a chemical inducer of dimerization [40].

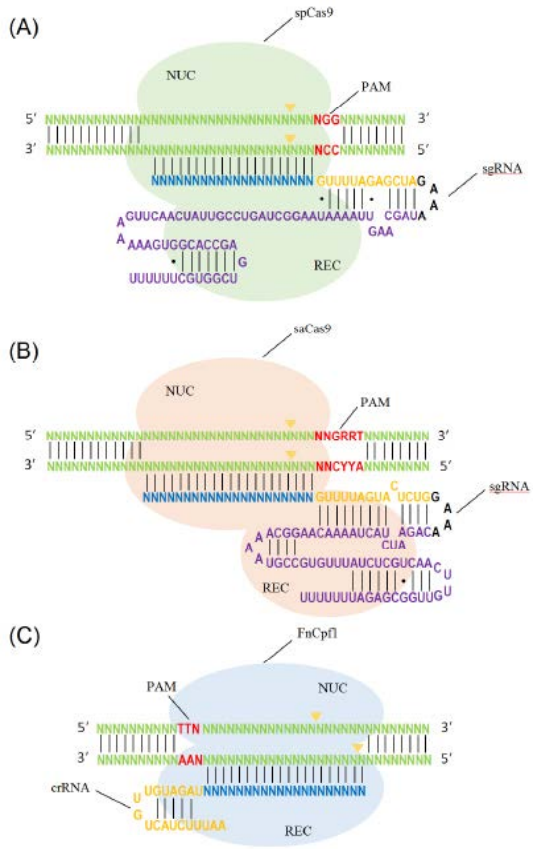

Figure 1: Schematics of the CRISPR-Cas complexes.

(A) **spCas9-sgRNA-DNA complex:** sgRNA consisting of a 20-nucleotide guide sequence (blue), a short direct repeat (orange), tetraloop (black) and tracrRNA (purple), directs spCas9 to a target DNA sequence followed by 5'-NGG PAM. spCas9 cuts 3 nucleotides upstream of the PAM sequence (cleavage site is shown with yellow triangle). (B) **saCas9-sgRNA-DNA complex**: sgRNA consisting of a 21-nucleotide guide sequence (blue), a short direct repeat (orange), tetraloop (black) and tracrRNA (purple), directs saCas9 to a target DNA sequence followed by 5'-NNGRRT PAM. Like spCas9, saCas9 cuts 3 nucleotides upstream of the PAM sequence (cleavage site is shown with yellow triangle). **(C) FnCpf1-crRNA-DNA complex:** crRNA, consisting of a 20-nucleotide guide sequence (blue) and a short direct repeat (orange), is sufficient for FnCpf1-mediated DNA cleavage. FnCpf1 cleaves target DNA distal to a 5'-TTN PAM (cleavage site is shown with yellow triangle).

Another important limitation of CRISPR-Cas9 system is on-target and off-target mutations caused by spCas9. It has been reported that spCas9 can be converted to DNA nickases by mutating certain aspartate (D10A) and histidine (H840A) residues of the RuvC and HNH catalytic domains, respectively. It has been shown that a Cas9-nickase (Cas9n) combined with paired sgRNAs resulted in a significant decrease (50 to 1500 fold) in off-target activity in cell lines [41]. More recently, Zhang and colleagues have published a modified spCas9, enhanced specificity spCas9, showing substantially decreased off-target cleavage, while retaining robust on-target cleavage activity [42]. This was soon followed by a modified spCas9 published by the Joung laboratory, where a different set of mutations resulted in substantially reduced off target cleavage while retaining robust on target nuclease activity [43].

The CRISPR-Cas9 system has been broadly used for gene editing in *in vitro* and animal models of human diseases. CRISPR-Cas9 has been demonstrated in several studies to efficiently correct dystrophin mutations in DMD patient iPSCs [42], DMD patients myoblasts [44], and in mouse models of muscular dystrophy [45,46]. In addition, CRISPR-Cas9-mediated HR has been successfully used to restore the cystic fibrosis trans membrane conductor receptor (CFTR) function in intestinal stem cells derived from cystic fibrosis patients [47] and correct β-thalassemia mutations in iPSCs derived from β-thalassemia patients [48]. In addition, CRIPSR-Cas9-mediated HR has been adopted to restore a Crygc mutation in a dominant cataract mouse model, resulting in the correction of cataract phenotype [49], and a Fan mutation in a mouse model of human hereditary tyrosinemia type I, which rescued the lethality in Fah-deficient mice [50]. CRISPR-Cas9 system has also been successfully harnessed to disrupt DNA viruses that cause chronic infection including HSV-1 [51], HIV [52,53] and HBV [54-56] *in vivo* and *in vitro*. For example, in one study CRISPR-Cas9, was able to induce the naturally occurring CCR5Δ32 mutation in wild-type iPSCs, resulting in resistance of the mutated iPSCs to HIV infection *in vitro* [30].

CRISPR-Cpf1

Cpf1 is a class II CRISPR single RNA-guided nuclease. Very recently, Cpf1from Francisella novicida U112 (FnCpf1) has been shown to effectively cut target DNA through a staggered double-stranded break. FnCpf1 is slightly smaller than spCas9 (1300 aa vs. 1620 aa). Like spCas9, FnCpf1 contains a predicted Ruv1-like domain but lacks a second HNH catalytic domain. Another advantage of FnCpf1 over spCas9 is that, unlike spCas9 that requires both crRNA and tracrRNA to mediate cleavage of a target DNA, crRNA is sufficient for FnCpf1-mediated DNA interference. The other difference between FnCpf1 and spCas9 is that spCas9 cleavage results in blunt ended DSBs, while FnCpf1 generates a 5-nucleotide 5' overhang upon DSB, providing a tool to precisely edit genome through non-HR mechanisms. In contrast to spCas9 which uses a G-rich PAM, FnCpf1 efficiently cleaves target sites followed by a T-rich PAM (5'-TTN) (Figure 1C) [57]. The relative HR vs error prone repair with the different Cas-9 systems and Cpf1 remain to be seen in therapeutically relevant cells, in order to allow their clinical translation.

Conclusion

The CRISPR-Cas system provides a robust and powerful tool for genome editing. However, in order to be used as a therapeutic tool, this technology will need a robust methodology to optimize its efficiency, specificity, and safety. In addition, novel approaches are needed to improve the rate of HR, which is necessary to make precise sequence changes and insertions.

References

1. Gaspar HB, Cooray S, Gilmour KC, Parsley KL, Adams S, et al. (2011) Long-term persistence of a polyclonal T cell repertoire after gene therapy for X-linked severe combined immunodeficiency. Sci Transl Med 3: 97ra79.
2. Aiuti A, Biasco L, Scaramuzza S, Ferrua F, Cicalese MP, et al. (2013) Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. Science 341: 1233151.
3. Cox DB, Platt RJ, Zhang F (2015) Therapeutic genome editing: prospects and challenges. Nat Med 21: 121-131.
4. Howe SJ, Mansour MR, Schwarzwaelder K, Bartholomae C, Hubank M, et al. (2008) Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. J Clin Invest 118: 3143-50.
5. Cavazzana-Calvo M, Payen E, Negre O, Wang G, Hehir K, et al. (2010) Transfusion independence and HMGA2 activation after gene therapy of human β-thalassaemia. Nature 467: 318-322.
6. Tiemann K, Rossi JJ (2009) RNAi-based therapeutics-current status, challenges and prospects. EMBO Mol Med 1: 142-151.
7. Stoddard BL (2011) Homing endonucleases: from microbial genetic invaders to reagents for targeted DNA modification. Structure 19: 7-15.

8. Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD (2010) Genome editing with engineered zinc finger nucleases. Nat Rev Genet 11: 636-646.

9. Bogdanove AJ, Voytas DF (2011) TAL effectors: customizable proteins for DNA targeting. Science 333: 1843-1846.

10. Hsu PD, Lander ES, Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. Cell 157: 1262-1278.

11. McVey M, Lee SE (2008) MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. Trends Genet 24: 529-538.

12. Davis AJ, Chen DJ (2013) DNA double strand break repair via non-homologous end-joining.Transl Cancer Res 2: 130-143.

13. Rothkamm K, Krüger I, Thompson LH, Löbrich M (2003) Pathways of DNA double-strand break repair during the mammalian cell cycle. Mol Cell Biol 23: 5706-5715.

14. Chapman JR, Taylor MR, Boulton SJ (2012) Playing the end game: DNA double-strand break repair pathway choice. Mol Cell 47: 497-510.

15. Chen F, Pruett-Miller SM, Huang Y, Gjoka M, Duda K, et al. (2011) High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. Nat Methods 8: 753-755.

16. Beumer KJ, Trautman JK, Mukherjee K, Carroll D (2013) Donor DNA Utilization during Gene Targeting with Zinc-finger Nucleases. G3 (Bethesda).

17. Epinat JC, Arnould S, Chames P, Rochaix P, Desfontaines D, et al. (2003) A novel engineered meganuclease induces homologous recombination in yeast and mammalian cells. Nucleic Acids Res 31: 2952-62.

18. DiCarlo JE, Chavez A (2015) Safeguarding CRISPR-Cas9 gene drives in yeast. Nat Biotechnol 33: 1250-1255.

19. Stoddard BL (2005) Homing endonuclease structure and function. Q Rev Biophys 38: 49-95.

20. Isalan M (2011) Zinc-finger nucleases: how to play two good hands. Nat Methods 9: 32-34.

21. Gabriel R, Lombardo A, Arens A, Miller JC, Genovese P, et al. (2011) An unbiased genome-wide analysis of zinc-finger nuclease specificity. Nat Biotechnol 29: 816-823.

22. Li H, Haurigot V, Doyon Y, Li T, Wong SY, et al. (2011) In vivo genome editing restores haemostasis in a mouse model of haemophilia. Nature 475: 217-221.

23. Genovese P, Schiroli G, Escobar G, Di Tomaso T, Firrito C, et al. (2014) Targeted genome editing in human repopulating haematopoietic stem cells. Nature 510: 235-240.

24. Holt N, Wang J, Kim K, Friedman G, Wang X, et al. (2010) Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted to CCR5 control HIV-1 in vivo. Nat Biotechnol 28: 839-847.

25. Perez EE, Wang J, Miller JC, Jouvenot Y, Kim KA, et al. (2008) Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. Nat Biotechnol 26: 808-816.

26. Crane AM, Kramer P, Bui JH1, Chung WJ, Li XS, et al. (2015) Targeted correction and restored function of the CFTR gene in cystic fibrosis induced pluripotent stem cells. Stem Cell Reports 4: 569-577.

27. Zou J, Mali P, Huang X, Dowey SN, Cheng L (2011) Site-specific gene correction of a point mutation in human iPS cells derived from an adult patient with sickle cell disease. Blood 118: 4599-4608.

28. Sebastiano V, Maeder ML, Angstman JF, Haddad B, Khayter C, et al. (2011) In situ genetic correction of the sickle cell anemia mutation in human induced pluripotent stem cells using engineered zinc finger nucleases. Stem Cells 29: 1717-1726.

29. Yusa K, Rashid ST, Strick-Marchand H, Varela I, Liu PQ, et al. (2011) Targeted gene correction of α1-antitrypsin deficiency in induced pluripotent stem cells. Nature 478: 391-394.

30. Tebas P, Stein D, Tang WW, Frank I, Wang SQ, et al. (2014) Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. N Engl J Med 370: 901-910.

31. Ousterout DG, Perez-Pinera P, Thakore PI, Kabadi1 AM, Brown MT, et al. (2013) Reading frame correction by targeted genome editing restores dystrophin expression in cells from Duchenne muscular dystrophy patients. Mol Ther 21: 1718-1726.

32. Bloom K, Ely A, Mussolino C, Cathomen T, Arbuthnot P (2013) Inactivation of hepatitis B virus replication in cultured cells and in vivo with engineered transcription activator-like effector nucleases. Mol Ther 21: 1889-1897.

33. Dreyer AK, Hoffmann D, Lachmann N, Ackermann M, Steinemann D, et al. (2015) TALEN-mediated functional correction of X-linked chronic granulomatous disease in patient-derived induced pluripotent stem cells. Biomaterials 69: 191-200.

34. Ma N, Liao B, Zhang H, Wang L, Shan Y, et al. (2013) Transcription activator-like effector nuclease (TALEN)-mediated gene correction in integration-free Î²-thalassemia induced pluripotent stem cells. J Biol Chem 288: 34671-34679.

35. Sun N, Zhao H (2014) Seamless correction of the sickle cell disease mutation of the HBB gene in human induced pluripotent stem cells using TALENs. Biotechnol Bioeng 111: 1048-1053.

36. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, et al. (2013) Genome engineering using the CRISPR-Cas9 system. Nat Protoc 8: 2281-2308.

37. Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, et al. (2015) In vivo genome editing using Staphylococcus aureus Cas9. Nature 520: 186-191.

38. Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, et al. (2015) An updated evolutionary classification of CRISPR-Cas systems.Nat Rev Microbiol 13: 722-736.

39. Truong DJ, Kühner K, Kühn R, Werfel S, Engelhardt S, et al. (2015) Development of an intein-mediated split-Cas9 system for gene therapy.Nucleic Acids Res 43: 6450-6458.

40. Zetsche B, Volz SE, Zhang F (2015) A split-Cas9 architecture for inducible genome editing and transcription modulation.Nat Biotechnol 33: 139-142.

41. Shen B, Zhang W, Zhang J, Zhou J, Wang J, et al. (2014) Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects.Nat Methods 11: 399-402.

42. Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, et al. (2016) Rationally engineered Cas9 nucleases with improved specificity. Science 351: 84-88.

43. Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, et al. (2015) Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature 523: 481-485.

44. Ousterout DG, Kabadi AM, Thakore PI, Majoros WH, Reddy TE, et al. (2015) Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause Duchenne muscular dystrophy. Nat Commun 6: 6244.

45. Nelson CE, Hakim CH, Ousterout DG, Thakore PI, Moreb EA, et al. (2016) In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. Science 351: 403-407.

46. Long C, Amoasii L, Mireault AA, McAnally JR, Li H, et al. (2016) Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. Science 351: 400-403.

47. Schwank G, Koo BK, Sasselli V, Dekkers JF, Heo I, et al. (2013) Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. Cell Stem Cell 13: 653-658.

48. Xie F, Ye L, Chang JC, Beyer AI, Wang J, et al. (2014) Seamless gene correction of β-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac.Genome Res 24: 1526-1533.

49. Wu Y, Liang D, Wang Y, Bai M, Tang W, et al. (2013) Correction of a genetic disease in mouse via use of CRISPR-Cas9.Cell Stem Cell 13: 659-662.

50. Yin H, Xue W, Chen S, Bogorad RL, Benedetti E, et al. (2014) Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype.Nat Biotechnol 32: 551-553.

51. Bi Y, Sun L, Gao D, Ding C, Li Z, et al. (2014) High-efficiency targeted editing of large viral genomes by RNA-guided nucleases.PLoS Pathog 10: e1004090.

52. Hu W, Kaminski R, Yang F, Zhang Y, Cosentino L, et al. (2014) RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. Proc Natl Acad Sci USA 111: 11461-11466.

53. Ebina H, Misawa N, Kanemura Y, Koyanagi Y (2013) Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus.Sci Rep 3: 2510.

54. Kennedy EM, Bassit LC, Mueller H, Kornepati AV, Bogerd HP, et al. (2015) Suppression of hepatitis B virus DNA accumulation in chronically infected cells using a bacterial CRISPR/Cas RNA-guided DNA endonuclease.Virology 476: 196-205.

55. Seeger C, Sohn JA (2014) Targeting Hepatitis B Virus With CRISPR/Cas9.Mol Ther Nucleic Acids 3: e216.

56. Lin SR, Yang HC, Kuo YT, Liu CJ, Yang TY, et al. (2014) The CRISPR/Cas9 System Facilitates Clearance of the Intrahepatic HBV Templates In Vivo.Mol Ther Nucleic Acids 3: e186.

57. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, et al. (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system.Cell 163: 759-771.