**Applications of Genome Editing Technology in Targeted Therapy of Human Diseases: Mechanisms, Advances and Prospects**

Hongyi Li1,#, Yang Yang1,#, Weiqi Hong2, Mengyuan Huang2, Min Wu3,\*, Xia Zhao1,\*

1 Department of Gynecology and Obstetrics, Development and related disease of women and children key laboratory of Sichuan Province, Key Laboratory of Birth Defects and Related Diseases of Women and Children, Ministry of Education, West China Second Hospital, Sichuan University, Chengdu, 610041, P. R. China.

2 Laboratory of Aging Research and Cancer Drug Target, State Key Laboratory of Biotherapy, National Clinical Research Center for Geriatrics, West China Hospital, Sichuan University, No. 17, Block 3, Southern Renmin Road, Chengdu, Sichuan 610041, PR China.

3 Department of Biomedical Sciences, School of Medicine and Health Sciences, University of North Dakota, Grand Forks, North Dakota 58203, USA.

Email addresses: Hongyi Li, [lihongyi961129@163.com](mailto:lihongyi961129@163.com); Yang Yang, [yang\_yang821@foxmail.com](mailto:yang_yang821@foxmail.com); Weiqi Hong, [923441488@qq.com](mailto:923441488@qq.com); Mengyuan Huang, [1076283043@qq.com](mailto:1076283043@qq.com); Min Wu, min.wu@UND.edu, Xia Zhao, xiazhaoscu@126.com.

# Contributed equally.

\*Corresponding authors: Min Wu, min.wu@und.edu (M.W.); Xia Zhao, xiazhaoscu@126.com;

Tel/Fax: 86-28-85502796

Address:20 South Renmin Road, Block 3, Chengdu City, 610041, Sichuan Province, People’s Republic of China.

**Abstract**

Based on engineered or bacterial nucleases, the development of genome editing technologies has opened up the possibility to directly target and modify genomic sequence in almost all eukaryotic cells. Genome editing has extended our ability to elucidate the contribution of genetics to disease by promoting the creation of more accurate cellular and animal models of pathological process, and has begun to show extraordinary potentials in a variety of fields, ranging from basic research to the application of biotechnology and biomedical research. Recent progress in developing programmable nucleases, such as zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)–Cas associated nucleases, have greatly expedited the proceedings of gene editing from concept to clinical practice. Here, we review recent advances of the three major genome editing technologies (ZFN, TALENs and CRISPR/Cas9) and discuss applications of their derivative reagents of gene-editing tools in various human diseases and potential future therapies, focusing on eukaryotic cells and animal models. Finally, we provide an overview of the clinical trials that apply genome editing platforms for disease treatment and some of the challenges in the implementation of this technology.

1. **Introduction**

Over the last few years, the exuberant development of genome editing has revolutionized research on human genome, which has enabled investigators to better understand the contribution of a single gene product to a disease in an organism. In the 1970s, the development of genetic engineering (manipulation of DNA or RNA) set up a novel frontier in genome editing.1 Based on engineered or bacterial nucleases, genome editing technologies have been developed at a tearing pace over the past 10 years and have begun to show extraordinary talents in various fields, ranging from basic research to the application of biotechnology and biomedical research.2 Genome editing can be achieved in vitro or in vivo by delivering the editing machinery in situ, which powerfully add, ablate and “correct” genes as well as perform other highly targeted genomic modifications.3,4 Targeted DNA alterations begin from the generation of nuclease-induced double-stranded breaks (DSBs), which leads to the stimulation of highly efficient recombination mechanisms of cellular DNA in mammalian cells.5,6 Nuclease-induced DNA DSBs can be repaired by one of the two major mechanisms that occur in almost all cell types and organisms: homology-directed repair (HDR) and nonhomologous end-joining (NHEJ),7 resulting in targeted integration or gene disruptions, respectively (**Figure 1**).

Historically, homologous recombination (HR), in which undamaged homologous DNA fragments are used as templates, has been the approach to realize targeted gene addition, replacement, or inactivation; however, the utility of HR is heavily limited due to its inefficiency in mammalian cells and model organisms.8 After it was discovered that DSB could raise the incidence of HDR by multiple orders of magnitude , targeted nucleases have been found as an alternative approach to increase the efficiency of HDR-mediated genetic alteration. Once a targeted DSB has been made, HDR may reconstruct the cleaved DNA using an exogenous DNA template analogue to the break site sequence.

This mechanism may be used to introduce precise mutations by delivering an appropriately designed repair template into targeted cells directly,9,10 thereby, in a site-specific manner, resulting in mutation correction or new sequence insertion. Alternatively, NHEJ–mediated repair tends to result in errors because it leads to efficient formation of gene insertion or deletion (indels) in diverse length at the DSB site, which eventually causes gene inactivation.11 If indels occur in the coding sequence, there will be frameshift mutations, which will result in mRNA degradation or non-functional truncated protein production by nonsense-mediated decay.12 This approach and its applications are thought to be simpler than HR-based methods because: (a) there is no need for a repair matrix; (b) the cell type has less impact on modification efficacy (contrary to HR, NHEJ may be active all through the cell cycle).13 Thus, similar to RNAi, NHEJ may be applied in immortalized cell lines to generate inactivation of a single gene or multiple genes, but by creating loss-of-function mutations it may lead to permanent gene inactivation.9

In early development stage of genome editing, to induce desired DSBs at each particular DNA target site, engineering of distinct zinc finger nucleases (ZFNs)14 or meganucleases15 has been the research focus. These nuclease systems required specialized competence to generate artificial proteins consisting of customizable sequence-specific DNA-binding domains, each connected to a non-specific nuclease for target cleavage, providing researchers with unprecedented tools to perform genetic manipulation.16 Subsequently, a new class of a Flavobacterium okeanokoites (FokI) catalytic domain derived from bacterial proteins termed as transcription activator–like effectors (TALEs) has shed light on new possibilities for precise genome editing.17 TALE-based programmable nucleases can cleave any DNA sequence of interest with relatively high frequency. However, the main challenges for TALEN approaches are the design of a complex molecular cloning for each new DNA target and its low efficiency of genome screening in successfully targeted cells.18 Clustered regularly interspaced short palindromic repeat (CRISPR)–CRISPR associated 9 (Cas9) nuclease is a recently discovered, robust gene-editing platform derived from a bacterial adaptive immune defense system.19 This system can be efficiently programmed to modify genome of eukaryotic cells via RNA-guided DNA cleavage module and has emerged as a potential alternative to ZFNs and TALENs to the induce targeted genetic modifications20 (**Table 1**). Since 2013 when it was first applied in mammalian cells as a tool to edit the genome,21,22 the versatile CRISPR/Cas9 technology has been rapidly expanding its use in modulating gene expression, ranging from genomic sequence correction or alteration, to epigenetic and transcriptional modifications.

The advent of programmable nucleases has greatly accelerated the proceedings of gene editing from concept to clinical practice and unprecedentedly enabled scientists with a powerful tool to maneuver literally any gene in a wide variety of cell types and species. Current preclinical research on genome editing primarily concentrate on viral infections, cardiovascular diseases, metabolic disorders, primary defects of the immune system, hemophilia, muscular dystrophy and development in T-cell based anti-cancer immunotherapies. Some of these methods have gone beyond preclinical research and are recently undergoing phase 1/2 clinical trials. Here, we review recent improvements of the three main genome editing platforms (ZFN, TALENs and CRISPR/Cas9) and discuss applications of their derivative reagents of gene-editing tools in various human diseases and in promising future therapies, focusing on eukaryotic cells and animal models. Finally, we outline the clinical trials that apply genome editing platforms for disease treatment and some of the challenges in the implementation of this technology.

1. **Structure and mechanism of genome editing tools**

**2.1 The structure of ZFN and its interaction with DNA**

Zinc-finger nuclease was assembled by fusing a none sequence-specific cleavage domain to a site-specific DNA binding domain that is loaded in zinc finger.23 The zinc-finger protein with site-specific binding property to DNA was discovered primarily in 1985 as part of transcription factor IIIa in Xenopus oocytes.24 Functional specificity of the designed zinc-finger domain comprises an array of Cys2His2 zinc fingers (ZFs), which are derived by highly conserved interactions of their zinc finger domains with homologous DNA sequences. Generally, an individual Cys2His2 zinc finger consists of approximately 30 amino acids, which constitute two anti-parallel β sheets opposing an α-helix.25 Cys2-His2-ZF is an adaptable DNA recognition domains and is considered to be the most common type of DNA binding motif in eukaryotes transcription factors.26 Each zinc finger unit selectivity recognizes 3 base pairs (bp) of DNA and produces base-specific contacts through the interaction of its α-helix residues with the major groove of DNA.27,28 The FokI type Ⅱ restriction endonuclease form the domain that cleave the DNA, which can be adopted as a dimer to directly target sequences within the genome for effective gene editing.29 Since the FokI nuclease needs to be dimerized to cleave DNA, two ZFN molecules are usually required to bind to the target site in an appropriate orientation,30 doubled in the number of specifically recognized bps. After DNA cleavage lead by ZFN is achieved in eukaryotic cells, DSB at a specific locus of the genome is initiated, creating the desired alternations in a subsequent endogenous NHEJ or HDR repair systems.23

Target sequence recognition and specificity of ZFN are determined by three major factors: (a) the amino acid sequence of each finger, (b) the number of fingers, and (c) the interaction of the nuclease domain. By the virtue of the modular structure of ZFNs, both the DNA-binding and catalytic domains can be individually optimized, which enables scientists to develop novel modular assembly with sufficient affinity and specificity for genome engineering. In early studies, individual ZFNs were used containing 3-6 fingers to interact with a 9-18 nucleotides target, which enabled ZFN dimers to specify 18-36 bp of DNA at each cleavage site.31 Since the 18 bp of DNA sequence can render specificity within 68 billion bp of DNA, this approach facilitated for the first time specific sequences to be targeted in the human genome. More recently developed strategy used architectural diversification to improve targeting accuracy of ZFNs via “selection-based methods”32: this study developed a new linker option for spanning finger–finger and finger–FokI cleavage domain junctions, which produced a 64-fold total increase in the number of ZFN configurations available for targeting cleavage to any given base of DNA.

**2.2 TALENs: protein-based DNA targeting system**

TALEN is another engineered nuclease that exhibits better specificity and efficiency than ZFN. Similar to ZFN, TALEN comprises a non-specific DNA cleavage domain fused to a customizable sequence-specific DNA-binding domain to generate DSBs. This DNA-binding domain consists of a highly conserved repeat sequence from the transcription activator-like effector (TALE), which is a protein originally discovered in the phytopathogenic Xanthomonas bacteria, naturally altering the transcription of genes in host plant cells.17,33 Binding of TALE to DNA is mediated by a central region which contains an array of 33- to 35-amino acid-sequence motif. The amino acid sequence of each repeat is structurally similar, except for two hypervariable amino acids (the repeat variable di-residues or RVD) at positions 12 and 13.34 DNA binding specificity is determined by RVD, with ND specifically binding to C nucleotides, HN to A or G nucleotides, NH to G nucleotides, and NP to all nucleotides.17 There is a one-to-one correspondence between RVDs and contiguous nucleotides in the target site, constituting a strikingly simple TALE–DNA recognition cipher.35

Functional endonuclease FokI is factitiously fused to DNA-binding domains to create site-specific DSBs and thereby stimulating DNA recombination, so as to achieve TALENs-induced targeted genomic modification. In order to cleave the two strands of the targeted DNA, the FokI cleavage domain must be dimerized. Hence, like zinc fingers, such a TALEN module is designed in pairs to bind opposing DNA target loci, with proper spacing (12–30 bp) between the two binding sites.36 However, as compared to zinc finger proteins, there is no need to redesign the linkage between repeats constituting long arrays of TALEs, which function to target individual genomic sites. Following pioneering works on zinc-finger proteins, multiple effector domains have become accessible to support the fusion of TALE repeats for different genomic modification purposes, including nucleases,37 transcriptional activators,18 and site-specific recombinases.38 Although their simpler cipher codes provide better simplicity in design than triplet-confined zinc-finger proteins, one of the primary technical hurdles for cloning repeat TALE arrays is the design of a large scale of identical repeat sequences. To conquer this limitation, a few strategies have been established to facilitate fast assembly of custom TALE arrays, including ‘Golden Gate’ molecular cloning,39 high-throughput solid phase assembly,40,41 and connection-independent cloning techniques.42

**2.3 CRISPR/Cas9: a versatile tool for genome editing**

Early in 1987, clustered regularly interspersed short palindromic repeats (CRISPR) were originally discovered in E. coli and later in many other bacteria species.43 The function of the short repeat sequences stayed unclear for many years before several studies in 2005 characterized their similarities to phage DNA, and subsequent experiments revealed that these sequences took part in bacterial and archaea adaptive immune defense against offending foreign DNA by inducing RNA-guided DNA cleavage.44-46 Generally, the CRISPR‐Cas systems are divided into two classes based on the structural variation of the *Cas* genes and their organization style.44 To be specific, class 1 CRISPR–Cas systems consist of multi-protein effector complexes, yet class 2 systems comprise only a single effector protein; altogether there have been six CRISPR–Cas types and at least 29 subtypes,47,48 and the list is rapidly expanding. The most frequently used subtype of CRISPR systems is the type II CRISPR/Cas9 system, which depends on a single Cas protein from Streptococcus pyogenes (SpCas9) targeting particular DNA sequences, therefore being an attractive gene editing tool.49 Mechanistically, the CRISPR/Cas9 system comprises two components, a single-stranded guide RNA (sgRNA) and a Cas9 endonuclease. The sgRNA often contains a unique 20 base-pair (bp) sequence designed to complement the target DNA site in a sequence-specific manner, and this must be followed by a short DNA sequence upstream essential for the compatibility with the Cas9 protein used, which is termed as the “protospacer-adjacent motif” (PAM) of an “NGG” or “NAG”.50,51 The sgRNA binds to target sequence by Watson-Crick base pairing and Cas9 precisely cleaves the DNA to generate a DSB.52 Following DSB, DNA-DSB repair mechanisms initiate genome repaired. With CRISPR/Cas9 system, through pathways of NHEJ or the high-fidelity HDR, targeted genomic modifications, including introduction of small insertions and deletions (indels) can be made.53

Known as the RNA‐guided system, CRISPR/Cas9 is more suitable for application compared to other gene editing technologies and has several important advantages.20 For example, endonuclease-based ZFN or TALEN tools demand reengineering of the enzyme to fit each target sequence and they should be synthesized separately for each case; however, the nuclease protein Cas9 is identical in all cases and can be conveniently engineered to recognize new sites via changing the guide RNA sequences (sgRNA), which match target sites by Watson–Crick base pairing. Moreover, in contrast to CRISPR/Cas9, ZFN and TALENs demand much more labor and are more expensive. An additional advantage of CRISPR/Cas9 is that it has the potential of simultaneous multiple loci editing, making this technology easier, more efficient, and more scalable compared to other genome editing technologies. CRISPR/Cas9 is now an indispensable tool in biological research.

Three common strategies have been developed for genome editing with the CRISPR/Cas9 platform: 1) the plasmid‐based CRISPR/Cas9 strategy, where a plasmid is used to encode Cas9 protein and sgRNA, 21,22 assemblies Cas9 gene as well as sgRNA into the same plasmid in vitro; this strategy is longer-lasting in the expression of Cas9 and sgRNA, and it prevents multiple transfections;54 However, the encoded plasmid needs to be introduced inside the nucleus of target cells, which is a key challenge in this system. 2) Direct intra-cellular delivery of Cas9 messenger RNA (mRNA) and sgRNA is the second strategy;55 The greatest drawback of this strategy lies in the poor stability of mRNA, which results in transient expression of mRNA and short duration of gene modification. 3) Directly delivery of Cas9 protein and sgRNA is the third strategy;56 this strategy owns several assets, including rapid action, great stability, and limited antigenicity.

The editing of DNA means the irreversible permanent change of genome information, and this process is also facing inevitable security risks and ethical problems. In addition, some cell types, such as neurons, are difficult to modify DNA using CRISPR/Cas9-mediated editing, which limits the use of gene therapy for nervous system diseases. As a result, genome editing strategies that only edit and modify RNA has also been proposed by scientists.57,58 As an intermediate product of DNA transcription, RNA is responsible for guiding the production of downstream proteins. With the use of CRISPR technology, RNA mutation is modified briefly, which not only avoids irreversible modification of genome, but also can repair protein function in almost all cells to treat a variety of diseases. Stem cell transplantation combining with CRISPR/Cas9 system is another approach for therapy of genetic mutations. It has been proved that patient-induced pluripotent stem cells (iPSCs) have the ability to differentiate into retinal precursors, and it is a useful cell source for cell replacement therapy without immune rejection problems.59,60 However, patient-derived iPSCs might still harbor the same pathogenic genes, which could influence the therapeutic efficacy of transplanted cells. Therefore, there is a necessity to combine CRISPR/Cas9 system to fix disease-causing mutations in patient-derived iPSCs before transplantation.61

1. **Genome editing for disease modeling and gene therapy**

Targeted gene modification via chimeric genome editing tools (eg, ZFN, TALEN and CRISPR/Cas9) is a powerful method to assess gene function and precisely manipulate cellular behavior and function. These genome editing tools have enabled investigators to use genetically engineered animal to understand the aetiology behind various diseases and to clarify molecular mechanisms that can be exploited for better therapeutic strategies (**Figure 2**).

**3.1 Cancer research**

Oncogenes and mutant tumor suppressor genes provide outstanding opportunities for the use of genome modulating approaches.62 Genome editing technology has accomplished crucial targeted cleavage events in various fundamental studies, from its initial proofs of efficient gene editing in eukaryotes to its recent applications in the engineering of hematopoietic stem cells (HSCs) and tumor-targeted T cells; this technology has established novel concepts of gene modification and has extended to a border field of cancer research.

As an archetypal platform for programmable DNA cleavage, ZFN-mediated targeting has been successfully applied to modify plenty of genes in human cells and a number of model organisms, thus opening the door to the development and application of genome editing technologies. ZFN-driven gene disruption was primarily demonstrated in 1994 when a three-finger protein was constructed to specifically block the expression of BCR-ABL human oncogene that was transformed into a mouse cell line.63 After that, a study used a human lymphoblast cell line derived from chronic myeloid leukemia (CML) patients, and a custom-designed ZFN was applied to this cell line to deliver site-specific DSB to the telomeric portion of the Mixed Lineage Leukemia (MLL) gene breakpoint cluster region as well as to analyze chromosomal rearrangements associated with MLL leukemogenesis via DSB error-repair.64 Successful targeted modulation was also achieved using designed ZFNs, which promoted the disruption of endogenous T-cell receptor (TCR) β- and α-chain genes. Lymphocytes treated with ZFNs lacked surface expression of CD3-TCR and expanded with an increase of interleukin-7 (IL-7) and IL-15.65 By targeting the promoter function of long terminal repeat (LTR) from human T cell leukemia virus type 1 (HTLV-1), a novel therapeutic ZFN specifically killed HTLV-1-infected cells in an in vivo model of adult T cell leukemia (ATL).66 In addition, it was reported that effective cleavage of BCR-ABL fusion gene by highly specific ZFN terminated translation of the BCR-ABL protein and induced apoptosis in imatinib resistant CML cells.67 Furthermore, cancer relevant translocations in human Ewing sarcoma and anaplastic large cell lymphoma (ALCL) cells induced by ZFNs demonstrated that precise genomic rearrangements can be achieved in relevant cell types by custom nucleases.68 Furthermore, the use of HER2 positive cell penetrating peptide (CPP) conjugated to mammalian mTOR-specific ZFN, has made the mTOR locus non-functional and inhibited relevant cancer signaling pathway, providing insight into the design of novel molecular targeted therapeutics for breast cancer (in particular) and other types of cancers.69 Moreover, as the tumor suppressor gene p53 plays a pivotal role in preventing cancer development, strategies of genome editing to restore wild-type p53 function have been investigated. A yeast-one-hybrid (Y1H) four-finger ZFN was designed to replace mutant p53 with wild-type p53 in several cancer cell lines (from glioblastoma, leukemia and breast cancer) via ZFN-induced HR.70 Although the HR events were not particularly effective in this case, modifications at p53 loci still provided framework for further investigation. In addition to modifying viral genes associated with tumorigenesis, researchers have applied ZFNs to optimize T cell mediated antitumor therapy. For example, by importing a chimeric TCR that comprises an extracellular IL-13 domain (zetakine) and a cytoplasmic CD3 domain into CD8+ T cells, glioblastoma-specific cytolytic T lymphocytes (CTLs) can be generated.. To achieve this goal, Reik et al.71 knocked down the glucocorticoid receptor in the modified CTLs with ZFN. Consequently, the cytolytic activity of “zetakine” transgenic CTLs against glioblastomas was preserved regardless of the presence of glucocorticoid treatment. This technology has recently been effective in knocking out glucose transport-related genes (MCT4 or BSG) in two glycolytic tumor models, colon adenocarcinoma and glioblastoma.72

A milestone of TALENs was achieved, when it was primarily applied to efficiently disrupt the endogenous genes NTF3 and CCR5 in human leukemia cells via introduction of NHEJ- or HDR-induced modification into coding sequence, demonstrating that TALENs could be designed for selective endogenous gene cleavage.73 Interestingly, when TALEN and ZFN were compared abreast at two human loci (CCR5 and IL2RG), TALENs showed a significant reduction in cytotoxicity. Moreover, the CCR5 specific TALEN was able to distinguish between the CCR5 target locus and a highly similar site in CCR2, when compared with ZFNs technology.37 By adopting TALEN gene-editing technology, precise disruptions have also been introduced into the T cell receptor α constant (TRAC) gene and the CD52 gene in allogeneic T cells by TALEN-induced HDR. The TALEN used in this study was engineered by a retroviral vector that expressed a chimeric antigen receptor (CAR) targeting CD19+ leukemic B cells, which helped to develop the “universal” CAR T cells (dKO-CART19).74 Alternatively, a site-specific TALEN was used to disrupt single allele of Fms related tyrosine kinase 3 (FLT3) gene and generate isogenic leukemia cell clones. TALENs-mediated FLT3 haplo-insufficiency impaired cell proliferation and colony formation in vitro. These suppressive effects were maintained in vivo and improved the survival rate of NOD/SCID mice transplanted with mutant K562 clones.75 The use of engineered TALENs in prostate cancer cells functionally classifies androgen receptor (AR) target genes rearrangements as drivers of resistance.76 Using TALENs to precisely cut the relevant translocation breakpoints, Piganeau et al. induced cancer-relevant translocations in anaplastic large cell lymphoma (ALCL).68 Through an analogous strategy, the reversion of ALCL translocation was achieved in a patient cell line, restoring the integrity of the two involved chromosomes. Recent studies have also shown that TALEN gene editing technology used to knock out genes in cancer cells (including cells from prostate cancer,76 breast cancer,77 hepatocellular carcinoma78) is a powerful and broadly applicable platform to explore gene mutations at molecular levels.

Because of its multiple advantages in genome editing, CRISPR/Cas9 system has attracted considerable attention and scientists gradually consider it as a powerful therapeutic tool for treating diseases associated with genome mutations. The ultimate goal of cancer therapy with CRISPR/Cas9 is to remove malignant mutations and replace them with normal DNA sequences.79 In a recent study, the leukemia model was generated by reviving several inactivated oncogenes through lentiviral delivery of the Cas9-sgRNA system in primary hematopoietic stem and progenitor cells (HSPCs).80 In this study, the pooled lentiviruses targeted genes including Tet2, Runx1, Dnmt3a, Nf1, Ezh2, and Smc3. Objective HSPCs were selected via a fluorescent marker; those HSPCs are engaged in the development of myeloid neoplasia. The CRISPR/Cas9 technology has also been adopted to establish organoid tumor models.81,82 For instance, organoid colon cancer models were constructed in vitro with CRISPR technology by introducing mutations of tumor suppressor genes (APC, TP53, SMAD4, etc.) and gene modification of oncogenes (KRAS, PI3K, etc.).83 Moreover, guided by colonoscopy, through mucosal injection, Roper et al.84 established CRISPR engineered mouse tumor organoids by delivering viral vectors carrying CRISPR/Cas9 components to the distal colon of mice. Such approach have already been applied in a study modelling tumor progression with adenoma-carcinoma-metastasis sequence. In the future, the use of CRISPR/Cas9 technology to establish precise cancer models shall significantly promote the research of functional cancer genomics and facilitate the advancement of cancer therapies.

**3.2 Cardiovascular Disease**

Cardiovascular disease (CVD) is a serious hazard to human health, and the number one killer in many industrialized countries. Many different types of CVD are usually associated with a single genetic mutation, or a combination of rare inherited heterozygous mutations.85 In practice, clinical treatments focus on the relief of disease symptoms without addressing potential genetic defects. Currently, the establishment of in vivo CVD disease models with gene editing technology and the in-depth analysis of CVD pathogenic genes as well as their molecular mechanisms, have made it possible to test the ability of gene therapy to control specific gene expression and improve gene functions. With the help of genome editing technologies, various research models of cardiovascular conditions have been created.

Abrahimi et al.86 used CRISPR/Cas9 to efficiently ablate major histocompatibility complex class II (MHCII) with double gene knockout in normal human endothelial cells. These cells retain the ability to form vascular structures without activating allogeneic CD4+ T cells. It is promising to apply such technology in the field of allograft bioengineering, including the refinement of heart transplant. In addition, the CRISPR/Cas9 technology can accurately remove β2M and CCR5 on CD34+ hematopoietic stem cells while retaining its ability of multi-differentiation, which provides possibility for future treatment of ischemic heart conditions with hematopoietic stem cells.86 In another study, Carroll et al.87 established cardiac-specific transgenic mouse model via injecting Cas9-containing plasmids into mouse zygotes; the expression of Cas9 was regulated by the Myh6 promoter. In this transgenic model, high levels of Cas9 was expressed exclusively in heart cardiomyocytes. The investigators then intraperitoneally injected sgRNA targeting Myh6 loaded in an adeno-associated virus (AAV) vector, subsequently inducing cardiac-specific gene modification at the Myh6 locus, finally leading to hypertrophic cardiomyopathy.

It has been demonstrated in the whole-exome sequencing of a nuclear family that three missense variants of single nucleotide in the MKL2, MYH7 and NKX2-5 genes pass on to three offspring of cardiomyopathy with childhood onset.88 Gifford et.al.89 adopted CRISPR/Cas9 to establish mouse model that encodes orthologous variants and showed that the complex of heterozygosity of all the three variants reproduced the phenotype of human disease. Analysis of mouse heart and human induced pluripotent stem cell-derived cardiomyocytes provides histological and molecular evidence for the contribution of the NKX2-5 variant as a genetic modifier.

Porcine models resemble human conditions by physiology, anatomy and genetics and are often considered ideal models for human cardiovascular structure research. Yang et.al.90 applied ZFN technology with nuclear transfer in somatic cells to generate endogenous gene knockout pigs, which have a specific mutation in peroxisome proliferator-activated receptor gamma (PARP-γ). Marfan syndrome (MFS) is an autosomal dominant disease caused by mutation of heterozygous fbrillin-1 (FBN1) with cardiovascular symptoms and skeletal abnormalities. By the same principle, Umeyama et al.91 accomplished the establishment of FBN1 mutant cloned pigs (+Glu433AsnfsX98), which exhibited phenotypes similar to those of humans with MFS, such as scoliosis, funnel chest, delayed epiphysis mineralization, and destruction of elastic fibers structure in the medial aortic tissue.

Human induced pluripotent stem cells (iPSCs) and CRISPR/Cas9 technology can also be combined to generate a congenital heart disease model associated with GATA4 mutations in vitro to investigate pathogenesis of this gene mutation.92,93 Using Barth syndrome (BTHS) iPSC-derived cardiomyocytes (iPSC-CMs) and genome editing, Wang and colleagues demonstrated that TAZ mutation is associated with myocardial metabolism, structural and functional abnormalities.93 These findings indicate the value of genetically edited animal as models for research on the pathogenesis of CVD and provide new insights in treatment strategies.

By genome editing techniques, potential therapeutic methods of repairing disease-causing mutations or of knocking out specific genes as CVD prevention approaches have also received widespread attention. For example, long QT syndrome (LQTS) is an autosomal dominant congenital heart disease. Hybrid mutations in multiple genes may lead to LQTS, some of which have relatively clear mutation sites with known molecular functions, such as hERG gene mutations in the pore-forming subunit alpha protein that encodes the potassium voltage-gated channel. The hERG gene mainly expresses and functions in cells of myocardium and smooth muscle, and its mutation can cause fatal ventricular arrhythmia.94 Repairing hERG gene mutations in cardiomyocytes using CRISPR technology may be an effective strategy to treat such LQTS.

Previous studies have noted that nonsense mutation carriers of proprotein convertase subtilisin/kexin type 9 (PCSK9) gene have significantly decreased levels of low-density lipoprotein cholesterol (LDLC) in their blood, compared with normal subjects (an allelic mutation corresponds to a 30% to 40% reduction).95 The blood level of triglyceride (TG) in subjects with nonsense mutation in apolipoprotein C3 (APOC3) genes significantly lower than that of normal people (an allelic mutation corresponds to a 40% decrease).96 The incidence of heart disease in both carriers was lower than that in normal subjects by more than 80%, suggesting that inhibition of PCSK9 and APOC3 gene expression can be used as a potential treatment for cardiovascular disease. Since these two genes are mainly expressed in liver cells, one idea is to directly introduce nonsense mutations to APOC3 or PCSK9 genes in liver cells through genome editing technology, thus to fundamentally inhibit protein synthesis and achieve long-term stable therapeutic effects.97,98

PRKAG2 cardiac syndrome is an autosomal dominant disease induced by a mutation in the PRKAG2 gene encoding AMP-activated protein kinase γ2 regulatory subunit. A recent study suggests that selective destruction of pathogenic mutations through CRISPR/Cas9 technology in vivo is an competent strategy to treat PRKAG2 heart syndrome and other dominant hereditary heart conditions.99

**3.3 Metabolic diseases**

Metabolic diseases refer to the pathological state in which the body's protein, fat, carbohydrates, etc. are metabolically disordered. It is a group of syndromes that caused by both genetic factors and the environment.100 The application of gene editing technology can be applied in functional gene screening, gene therapy and construction of metabolic disease models, such as obesity, diabetes and hyperlipidemia. Leptin (Lep) is a hormone secreted by white fat cells that acts on the metabolic regulation center of the hypothalamus through the leptin receptor (LepR).101 It has diverse functions, including appetite suppression, energy intake reduction, fat synthesis inhibition, and can regulate blood sugar concentration, neuroendocrine etc. A number of animal models have been developed to illustrate the important role of Lep/LepR in glycolipid metabolism, and the most widely used are ob/ob mice against Lep and db/db mice against LepR.102 Chen and colleagues injected TALENs components into rat zygotes to specifically knockout LepR, thus obtaining three lines of rats with LepR mutations.103 Phenotypes in these strains manifested as obesity and other metabolic disorders; besides, the authors established a LepR mutant obese rat model, exhibiting efficient germline transmission. Bao et al.104 successfully established LepR knockout mice using CRISPR/Cas9 technology. The homozygous LepR-deficient mice are characterized by obesity, hyperphagia, hyperglycemia, insulin resistance and lipid metabolism disorders, together with some complications of diabetes. The same principle has been used to generate the cytochrome P450 (CYP) 2E1 knockout rat model with CRISPR/Cas9 technology to explore the role of CYP2E1 gene in biochemical metabolism, toxicology, and diseases (e.g. diabetes and alcoholic cirrhosis).105 The FTO allele is associated with obesity, which inhibits the mitochondrial thermogenic effects in adipose precursor cells. FTO gene mutations inhibit the conversion of white fat to brown fat. The FTO gene-regulated thermogenic pathway involves ARID5B, rs1421085, IRX3, and IRX5 factors. The rs1421085 can be edited using the CRISPR/Cas9 platform to repair the pattern structure of ARID5B, thereby suppressing the expression of IRX3 and IRX5, meanwhile achieving the effect of weight loss.106

As an important “experimental tool”, the animal model of diabetes can be used for pathological observation, preclinical experiments and drug screening. In a study based on CRISPR/Cas9 technology, pX330 (containing gRNA and Cas9 sequences together with the donor DNA plasmid) was injected into the oocyte to generate new Cre tool mice and achieve genetic manipulation of pancreatic β cells.107 The Ins1 (insulin gene) promoter and stop codon sequences served as targets for recombinase Cre insertion. Progeny F1 mice were histologically labeled as Cre-loxP recombination, which was observed in all islets expressing insulin-positive cells and negatively expressed in other tissues. There was no significant difference in glucose tolerance between these genetically edited mice and wild type mice. Applying CRISPR/Cas9 technology in human iPSCs to target diabetes-related genes has become a promising approach to explore the molecular mechanisms of diabetes. For example, human iPSCs are isolated from single-gene diabetic MODY patients, and genes encoding possible mutations such as HNF4A, GCK, PDX-1, and INS are edited by CRISPR; the edited iPSCs then differentiate into pancreatic progenitor cells and are later transplanted into patients.108 In addition, gene editing tools can also structurally modify proteins that promote chromatin structural variation, such as methylase, demethylase, acetylase or deacetylase, to treat diabetes epigenetically.109

Gene editing technology is also critically involved in the study of lipid metabolism.110 The cAMP responsive element binding protein 3-like 3 (CREB3L3), a transcription factor expressed in the liver and small intestine, controls the energy metabolic equilibrium in fasting response. Nakagawa et al.111 used the one-step CRISPR/Cas9 system to establish the CREB3L3-floxed murine model for the first time, and subsequently obtained mice that were knocked out of CREB3L3 gene in the small intestine and liver, respectively. Evidence above provides new understanding on the role of CREB3L3 in plasma triglyceride metabolism and the its contribution in liver and intestinal cholesterol metabolism. Familial hypercholesterolemia is an autosomal single-gene dominant disease correlated with a defect in the low-density lipoprotein receptor (LDLR) gene, which causes a disorder in the body's lipid metabolism. In 2012, Carlson et al.112 used TALENs technology to target LDLR in porcine fetal fibroblasts, and obtained miniature swine containing mono- and biallelic mutations in LDLR, thus generating models of familial hypercholesterolemia, which came with critical biomedical significance in simulating lipid metabolic syndrome. Recent genome-wide association studies have identified tribbles homolog 1 (TRIB1) to be associated with lipoprotein metabolism in human hepatocytes. Hepatic-specific overexpression of Trib1 reduced plasma TG and cholesterol levels by reducing the production of VLDL; in contrast, Trib1-knockout mice showed elevated plasma TG and cholesterol levels due to increased production of VLDL.113 To further explore its regulation on lipid metabolism, Nagiec et al.114 induced destruction to the chromosome at the TRIB1 locus by delivering CRISPR/Cas9 system into mouse liver via a non-pathogenic AAV, which increased the transcription of PCKS9 and the secretion of PCKS9 protein; these responses ultimately reduced the level of liver LDL receptors and increased the level of LDL-C in the blood.

**3.4 Neurodegenerative Diseases**

Neurodegenerative diseases (ND), at least including Huntington's disease (HD), Alzheimer's disease (AD), and Parkinson's disease (PD) , have become a group of conditions attracted most worries for there have been no specific diagnostic approaches or established treatments for them.115,116 There are a few potential pathogenic mechanisms behind NDs, including accumulation of protein with abnormal structures ,117 impaired ubiquitin-proteasome and/or autophagic lysosomal pathways,118 oxidative stress119 and circuit alternations120 etc. These mechanisms indicate that NDs are induced by complicated interactions of multiple genetic factors; either alone or in combination, the interactions lead to clinical features. The emergence of gene editing platforms provides a convenient approach to study gene functions related to NDs.121

In HD, in vitro investigations demonstrated that via ZENs chromosomal expression of the mutant Huntington gene (HTT) was significantly reduced at both the protein and mRNA levels; in vivo studies revealed that via striatal AAV delivery into the HD R6/2 mice ZFNs extensively suppressed cerebral expression of the HTT gene and ameliorated HD-related symptoms.122 Also, the HTT exon 1 in human iPSCs derived from fibroblasts of HD patients (HD-iPSCs) can be corrected by TALENs.123,124 To better understand the pathogenesis of HD, Yan et al.125 adopted CRISPR/Cas9 to establish a genome edited porcine model of HD in 2018, which internally expressed full-length mutant HTT. Being a promising breakthrough in the field of NDs, the development of HTT gene knock-in pigs would be of great significance for pathogenesis research and therapy exploration in Huntington disease.

Mutations in the gene encoding amyloid precursor protein (APP) cause familial AD with nearly complete penetrance.126 Mouse fibroblast cells could overexpress APP by receiving electroporated ZFNs that designed with DNA fragment containing the promoter and the protein coding regions of APP. These transgenic cells can be used to elucidate aspects of the molecular mechanisms of AD pathogenesis, particularly those involved in the mutant amyloidogenic pathway affecting the APP coding sequence.127 The A673V variant near APP β-secretase cleavage site, contributes to AD pathology via increasing Aβ and enhancing its aggregation as well as toxicity;128 on the contrary, the A673T variant, which is adjacent to the aspartyl protease β-site in APP, provides protection against AD progression.129 When A673V and A673T were induced in normal iPSCs by TALEN technology, these cells differentiated and form cortical neurons, presented with different levels of AD-associated biomarkers.130 In addition, through a gene editing platform based on single-stranded oligonucleotide DNA nucleotides and CRISPR/CAS-blocking mutations, Paquet et al.131 generated human iPSCs with dominant AD-causing mutations in APP and presenilin 1 (PSEN1), both heterozygous and homozygous , leading to early disease onset; thereby they yielded cortical neurons, which showed genotype-dependent phenotypes associated with AD. Apolipoprotein E4 (APOE4) is a genetic risk factor for late onset AD, while ApoE2, with only two bases (two bases C in APOE4, corresponding to two bases U in APOE2) different from APOE4, is not a risk factor for AD. Zhang and his team has introduced APOE4 RNA related to disease risk into cells, and successfully changed APOE4 to APOE2 sequence through RESCUE (RNA Editing for Specific C to U Exchange) editing system by changing two bases of C in APOE4, which is equivalent to converting the disease risk of AD high-risk population carrying APOE4 gene to zero.132

Alpha synuclein (SNCA) and leucine-rich repeat kinase 2 (LRRK2) are associated with autosomal dominant PD, whereas another group of genes are associated with autosomal recessive PD, including Parkin, phosphatase and tensin homolog–induced kinase 1 (PINK1), DJ-1, and ATPase type 13A2 (ATP13A2).133 The missense mutation of SNCA and LRRK2 genes can be corrected by ZFNs in vitro. After correction, the mtDNA damage disappeared in differentiated neural progenitor and neural cells derived from iPSCs.134,135 Additionally, Soldner et al.136 combined genome-wide epigenetic information with CRISPR/Cas9 genome editing to generate a genetically precisely controlled experimental system in Human iPSCs. This system has identified PD-associated risk variants in non-coding distal enhancer elements that regulate SNCA expression; it has also confirmed that the transcriptional disorder of SNCA is related to sequence-dependent binding of the brain-specific transcription factors EMX2 and NKX6-1. These results suggest that gene editing techniques can generate specific ND animal models for further exploration into human diseases and they are potentially capable of offering robust therapeutic approach against multiple human genetic defects that have been considered incurable.

**3.5 Viral infected diseases**

Gene editing platforms have emerged recently as anti-viral therapeutics for treating infectious diseases, either by altering host genes required by the virus, or by targeting viral genes necessary for replication.137 To date, genome editing-based HIV therapy has involved the modifying infection-related genes to produce HIV resistant CD4+ T cells and subsequent reinfusing the edited cells into patients. In 2008, the anti-HIV efficacy of ZFN system was first presented in preclinical studies by adopting primary human CD4+T cells.138 Approximately 50% of the CCR5 alleles were disrupted with ZFN, which was delivered by the chimeric Ad5 / F35 adenoviral vector. HIV-infected mice transfused with ZFN-modified CD4+ T cells also better preserved their original CD4+ T cells and had lower viral loads. In 2009, a patient was functionally cured of HIV infection by transplanting allogeneic stem cells from a donor with homozygous CCR5 d32 allele,139 suggesting that it is feasible to obtain resistance to HIV by mimicking natural homozygous CCR5 d32 mutation with genome editing technologies. In addition, engineering CD34+ HSPCs instead of CD4+ T cells with CCR5 ZFN pair provides a durable source of modified cells and also protects the CD4+ myeloid cells that are susceptible to HIV-1 as well.140 Further in vivo experiment showed that mice transplanted with ZFN-modified HSPCs experienced rapid selection for CCR5(-/-) cells, which had obviously lower levels of HIV-1 than the control group and maintained humans cells throughout their tissues. Disruption of C-X-C chemokine receptor 4 (CXCR4) is also under exploration as a strategy for patients who harbor CXCR4-tropic HIV-1.141 Simultaneous genetic inactivation of both CCR5 and CXCR4 in human CD4+ T cells by ZFNs confers protection against viruses that exclusively use the targeted coreceptor.142 Nuclease platforms based on TALEN143 and CRISPR/Cas9144-146 are also being applied to disrupt CCR5 in T cells and HSPCs. Laboratory results from Ebina and Hu et al.144,147 showed that CRISPR/Cas9 could not only specifically eradicate latent HIV infection, but also prevent new HIV infection. Similarly, Hendel et al.146 recently demonstrated that co-delivery of chemically modified CCR5 sgRNA with Cas9 mRNA/protein enhanced genome editing efficiency of human primary CD4+ T cells and CD34+ HSPCs, with no DNA-delivery-associated toxicity.

Sustained expression of high-risk human papillomavirus (HPV) oncogenes E6 and E7 is implicated in malignant transformation and is strongly associated with cervical cancer.148 Targeting mutagenesis of those high-risk HPV genes by gene editing tools may be a potential genetic therapy and may reverse cervical cancer in-situ. Ding et al.149 constructed a ZFN that could specifically recognize and cleave HPV16/18 E7 DNA. In their study, ZFNs-mediated HPV16/18 E7 DNA disruption directly decreased the expression of E7, which resulted in efficient growth inhibition and type-specific apoptosis in HPV16/18-positive cervical cancer cells in vitro. When different plasmids-encoded zinc finger modules were introduced in vivo, the therapeutic effects of ZFNs were further confirmed, inhibiting tumor growth in mice bearing cervical cancer cells. Similar results in another study showed that using ZFNs to target HPV E7 induced specific shear of the E7 gene and attenuated its malignant biological effect.150 Wayengera et al.151 computationally generated paired zinc finger arrays (pZFA) to target and cleave genomic DNA of HPV-type 16 and 18 respectively. The authors highlighted the therapeutic effect of ZFN-mediated gene disruption in HPV 16/18, which was achieved when HPV-derived viral plasmids or vectors were introduced into precancerous lesions to realize target-mutagenesis and gene-therapeutic reversal of cervical neoplasia. Additionally, combined treatment of ZFNs with two chemotherapeutic drugs (cisplatin and trichostatin A) increased apoptotic rate by about two times more than that of ZFNs used alone in HPV16/18-positive cervical cancer cells. Both of the chemotherapeutic drugs coordinated with ZFNs to downregulate HPV16/18 E7 expression while elevate retinoblastoma 1 (RB1) expression.150 TALEN-mediated targeting of HPV oncogenes E6 and E7 within host DNA resulted in the restoration of host tumor suppressors p53 and RB1, which not only reduced tumorigenicity in HPV-positive cell lines and ameliorated HPV-related cervical malignancy in transgenic mouse models.152 Furthermore, CRISPR‐Cas9/HPV16 E6/E7 sensitized cervical cancer cells to cisplatin, indicating the potential of application in cervical cancer therapy.153

Hepatitis B virus (HBV) is the most important pathogen of liver disease. Co-transfection of engineered ZFN pairs with a target plasmid containing the HBV genome results in specific cleavage.154 Rananan et al.155 designed and screened an efficient gRNA targeting the HBV genomic locus and transmitted the sgRNA/Cas9 system by lentiviral vector to HepG2 cells that were integrated with HBV. Finally, the amount of covalently closed circular DNA (cccDNA) gradually decreased, dropping by 92% on the 36th day; besides, HBV gene expression and replication were also inhibited. One study also attempted to knock out Epstein-Barr virus (EBV)-related genes using CRISPR/cas9 technology to treat latent infections caused by EBV.156 They used a plasmid containing CRISPR/cas9 to treat Raji cells isolated from Burkitt's lymphoma with EBV latent infection; then they found that cell proliferation was significantly inhibited and intracellular EBV load was significantly reduced.

Genomic editing technology allows us to gain a deeper understanding of the mechanisms underlying variant diseases associated with viral infection and it demonstrates tremendous potential in the development of therapeutic approaches against viral infections, one of the most intractable diseases..

**3.5 Hereditary eye diseases**

In recent years, with the advancement of gene sequencing technology, it is more explicit to make genetic diagnosis of a variety of hereditary eye diseases, such as congenital cataract, congenital glaucoma, retinitis pigmentosa (RP), congenital corneal dystrophy, Leber congenital amaurosis (LCA), retinoblastoma (RB) and Usher syndrome.157

CRISPR/Cas9 has already been used to generate animal models of RP. Receptor Expression Enhancer Protein 6 (REEP6), a member of REEP/Yop1 family proteins, influences the structure of endoplasmic reticulum.158 Arno et al. has reported that biallelic mutations in REEP6 cause autosomal-recessive retinitis pigmentosa.159 They identified variants in REEP6 in patients with RP from unrelated families. Moreover, they created a knock-in mouse model of Reep6 p.Leu135Pro via CRISPR/Cas9. The clinical phenotypes of RP were replicated in the Reep6L135P/L135P homozygous knock-in mice, such as developing photoreceptor degeneration and dysfunction of the rod photoreceptors, which provides a better animal model for future studies of RP. Rodless (rd1) mouse, the most vastly used preclinical model of RP, has been aggressively debated for nearly a century after its occurrence because the cause of the blinding RP phenotype remains undetermined. The rd1 mouse has two homozygous variants in Pde6b locus of chromosome 5: a nonsense mutation (Y347X) and a murine leukemia virus (Xmv-28) insertion in the reverse orientation in intro 1.160,161 Wu et al. repaired the nonsense point mutation via CRISPR/Cas9 to rescue and ameliorate the disease, demonstrating that Y347X mutation in rd1 mice is pathogenic.162 Another animal model of RP, the transgenic S334ter-3 rat possesses the mutation RhoS334, which shows similar phenotypes to human class- I RHO mistracking mutations, leading to continual degeneration of photoreceptors and vision decline.163,164 The protospacer adjacent motif (PAM) sequence in RhoS334 (5′-TGG-3′) diverges from the PAM in RhoWT (5′-TGC-3′) by only one nucleotide. Benjamin et al. reported that allele-specific disruption of RhoS334 via a single subretinal injection of CRISPR/Cas9 and gRNA by electroporation prevented retinal degeneration, and increased visual acuity.165 Additionally, Latella et al. successfully edited the human Rhodopsin (RHO) gene by electroporation of plasmid-based CRISPR/Cas9 in P23H transgenic mouse model for autosomal dominant RP, and confirmed its efficacy as a genetic engineering tool in photoreceptor cells,166 which strongly demonstrates that CRISPR/Cas9 system is an efficient and promising therapeutic tool for retinal degeneration such as RP. Suzuki et al. also figured out a CRISPR/Cas9 mediated homology-independent targeted integration (HITI) strategy, and demonstrated its efficacy in ameliorating visual function in a rat model of RP.167 HITI is a targeted integration mediated by NHEJ and this study is the first time that HITI could play a role in non-mitotic cells. The advantage of HITI technology is that it can be applied to any targeted genome engineering system, not just CRISPR/Cas9.

Combination of CRISPR/Cas9 technology and other methods provides new avenues for the treatment of related eye diseases, such as AAV and iPSCs. Bassuk et al. firstly reported that CRISPR/Cas9 precisely repair retinitis pigmentosa GTpase regulator (RPGF) point mutation, which causes X-linked RP in patient-specific iPSCs; this supports that combining gene editing with autologous iPSCs could be a personalized iPSCs transplantation strategy for therapies of various retinal degeneration.168 Similarly, Deng et al. found that iPSC-derived retinal organoids from three RP patients with different frameshift mutations in RPGR gene have significant defects in photoreceptor, including morphology, localization, and electrophysiological activity. Correction of RPGR mutation via CRISPR/Cas9 reverses ciliopathy and rescues photoreceptor loss, which indicates CRISPR/Cas9 to be an adopted mutation repair strategy.169

LCA is a congenital retinal dystrophy, which causes significant vision loss at an early age.170 To verify that mutation in human KCNJ13 causes LCA, Zhong et al. employed CRISPR/Cas9 to create Kcnj13 mutant mice by zygote injection with sgRNA and spCas9 mRNA. Kcnj13 mutant mice showed declined response to light, loss of photoreceptor and rhodopsin mislocalization, revealing that loss of Kcnj13 function could mimic human LCA phenotypes in mice.171 As demonstrated by Zhong et al., CRISPR/Cas9 could accelerate the study of candidate gene function in biology and disease.171 Centrosomal protein 290 kDa (CEP290) gene, the most frequent mutation in LCA, causes the most common subtype of LCA that is referred to as LCA10. However, the large size of CEP290 exceeding the capacity of AAVs delivery prevents the use of this delivery platform. To overcome this capacity limitation, Ruan et al. used a dual recombinant AAV vectors to induce CRISPR/Cas9 mediated deletion of a specific intronic fragment of Cep290 gene in mouse photoreceptors.172 Additionally, using smaller S. aureus CRISPR/Cas9 system enables a single AAV vector to deliver Cas9 gene and two gRNAs, which performs a dual-cut excision of the CEP290 mutation-containing region in primary fibroblasts from LCA10 patient.164 Recently, Maeder et al. developed a candidate genome-editing therapy named EDIT-101 to restore vision loss in LCA10.173 They delivered the Staphylococcus aureus Cas9 and CEP290 gRNA to photoreceptor via an AAV5 vector. Humanized CEP290 mice showed rapid and continuing CEP290 gene editing after subretinal delivery of EDIT-101. These extraordinary studies provide a roadmap for the preclinical advance of gene therapy for LCA10.

RB is the most common pediatric eye tumor of the developing retina.174 About 1/3 of RB cases are caused by bi-allelic RB1 mutation or deletion. Solin SL et al. has reported that using TALEN gene editing to inactivate somatic rb1 in adult zebrafish induced tumorigensis at high frequency.175 A highly penetrant and rapid RB preclinical model was reported by Naert et al., utilizing CRISPR/Cas9 system to induce knockout of rb1 and retinoblastoma-like 1 (rbl1) in Xenopus tropicalis.176 The animal model showed rapid development of RB, and it will be a good model for early stage drug discovery and rapid therapeutic target identification. Jian Tu et al. generated a pluripotent H1 human embryonic stem cell line with RB1 heterozygous knockout by CRISPR/Cas9 nickase, which provides a valuable cell resource for the study of hereditary retinoblastoma.177 Galucoma is the second leading cause of blindness worldwide, characterized by elevated intraocular pressure (IOP).178 Gain-of-function mutations in myocilin (MYOC) have been reported to commonly cause primary open-angle glaucoma (POAG).179-181 The accumulation of mutated myocilin inside cell leads to activation of unfolded protein response (UPR) cascade and endoplasmic reticulum (ER) stress in the trabecular meshwork (TM).182,183 TM cells are sensitive to chronic ER stress and finally die, resulting in increased IOP and glaucoma.184,185 Jain et al. knocked down the expression of mutant MYOC in a mouse model of POAG by CRISPR/Cas9, resulting in reduction of ER stress, lower IOP, and preventability of further glaucomatous damage in mouse eyes.186 Importantly, they also demonstrated the feasibility of utilizing CRISPR/Cas9 in human eyes for glaucoma. A dominant-negative mutation in KRT12,187 which causes Meesmann’s epithelial corneal dystrophy (MECD), results in the occurrence of a novel Streptococcus pyogenes PAM. Courtney et al. designed a sgRNA complementary to the sequence adjacent to this PAM, and found this sgRNA has high effect on decrease in mRNA and protein of KRT12 in vitro.188 Injection of combined Cas9/sgRNA into the corneal stroma of a humanized MECE mouse model showed frame-shifting deletions of the mutated KRT12 allele. This study is the first to demonstrate in vivo allele-specific CRISPR/Cas9 gene editing of a novel PAM created by a heterozygous disease-causing SNP.188

**3.6 Hematological Diseases**

Nearly half of hemophilia A cases are caused by inaccurate expression of factor VIII (F VIII) due to inversion of the chromosome.189 In one study, iPSCs were derived from somatic cells of hemophilia A patients induced by chromosome inversion, and the F VIII gene of iPSCs was modified by CRISPR/Cas9 technology.190 The modified iPSCs were induced to differentiate into mature endothelial cells capable of expressing factor VIII, and then transplanted into hemophilia mice lacking factor VIII. The results showed that the transplanted mice began to produce factor VIII, which effectively inhibited bleeding symptoms. Hemophilia B is caused by a deficiency in factor IX (F IX). Coagulation activity can be restored by increasing FIX in plasma. Guan et al.191 found that the F9 gene carries a new mutation, Y371D in a family of hemophilia B patients, which leads to a more severe hemophilia B phenotype than the previously discovered Y371S mutation. They used naked DNA constructs and adenoviral vectors to deliver Cas9 to adult F9 Y371D mutant mice, respectively. After treatment, it was found that when adenovirus was used as a vector to deliver cas9, although the mutation gene was highly efficiently repaired, hepatotoxicity was severe. On the other hand, Cas9 with naked DNA structure successfully repaired more than 0.56% of F9 alleles in hepatocytes in hemophilia B mice, enough to restore hemostasis. The CRISPR technology also provides a quick path to build hemophilia models. Researchers from the Institute of Zoology in the Chinese Academy of Sciences injected the CRISPR/Cas9 system targeting vwF (vascular hemophilia mutant gene) into the fertilized eggs of miniature pigs, and obtained the double allele mutant mini-pig quickly and efficiently. These miniature pigs have severe coagulopathy, indicating the successful construction of a miniature pig model of von Willebrand disease by CRISPR technology.192

Sickle anemia is the first genetic disease with an clearly understood pathogenesis. A single nucleotide mutation from A to T in the first exon of human β-globin results in a lesion.193 In 2016, the Stanford University team reported on the use of CRISPR/Cas9 technology to repair β-globin gene (HBB) mutations in patient-derived hematopoietic stem cells in vitro.194 After the modified iPSCs differentiated into red blood cells, normal HBB mRNA could be detected. This preclinical experiment provided theoretical support for gene editing technology in the treatment of sickle anemia. The CRISPR/Cas9 system has also been used to correct β thalassemia-causing mutations in the HBB.195 Using CRISPR/Cas9 technology to direct the calibrated DNA sequence to the HBB mutation site, it was possible to correct two different β-thalassemia mutations in the HBB gene of patient iPSCs by HR.

**3.7 Other hereditary disease**

Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy caused by mutations of DMD gene.196 Current X-linked muscular dystrophy (mdx) mice can only partially mimic human disease conditions. Their small size, limited chronic muscle damage and muscle weakness also impose limitations on disease research and analysis. Considering of this, larger ones such as rats, rabbits or pigs are more valuable for preclinical studies. Larcher et al.197 generated Dmdmdx rats by targeting exon 23 of DMD with TALENs. These edited rats showed a significant reduction in muscle strength and decreased spontaneous motor activity. Sui's team generated DMD knockout rabbits by co-injecting Cas9 mRNA and sgRNA into rabbit zygotes targeting exon 51 of DMD. These rabbits harbored typical phenotypes of DMD and the pathological features in the diaphragm and heart were similar to those of DMD patients.198 Meanwhile, the monkey dystrophin gene was targeted using CRISPR/Cas9 to create mutations that cause DMD. Detection of relative targeting rate showed that CRISPR/Cas9 could result in mosaic mutations in up to 87% of the dystrophin alleles in monkey muscle.199,200 Notably, three groups of researchers have recently described the use of CRISPR/Cas9 to remove mutations in the DMD gene encoding dystrophin, which affects protein expression.201-203 The investigators used the CRISPR/Cas9 system to excise the mutant portion of DMD in mdx mice model, thereby synthesizing a shorter version of dystrophin protein in the muscle fibers and restoring partial muscle function. This provided a promising method for correcting disease-causing mutations in the muscle tissue of patients.

Patients with primary immunodeficiencies have partial immune system missing or dysfunction, and they can be treated with allogeneic hematopoietic stem cells (HSCs) transplantation.204 This may be a high-risk process when leukocyte antigen-matched donors lack tissue compatibility. Correcting patient's own hematopoietic stem cells through gene therapy provides an attractive option. They can also be used in situ to correct pathogenic mutations and to develop cell or animal models to study the pathogenic effects of specific genetic defects found in immunodeficient patients. As the most severe immunodeficiency, severe combined immunodeficiency (SCID) is caused by a mutation in the gene encoding the interleukin 2 receptor gamma (IL2RG) that result in developmental arrest of T cell production and additional primary or secondary defects in B cells. Several research teams have successfully used ZFNs and TALENs techniques to induce HDR at the IL2RG locus in various human cell types, including HSCs and embryonic stem cells (ESCs).205-207 Other studies have utilized endonucleases to generate different kinds of immunodeficient animal models that were previously unable to establish due to lack of effective genetic modification.207-210 As a result of engineered nucleases-mediated editing of genomic modifications, other animal disease models have been developed, simulating Rett syndrome,211 hereditary deafness,212 Wilson disease,213 Laron syndrome,214 Niemann-Pick disease,215 Netherton syndrome216 and so on. Advances in genome editing technologies will further expand the application of animal models in disease mechanism research and treatment development.

1. **Future application prospects**
   1. **Genome editing in cancer immunotherapy**

Recently, cancer immunotherapy has stimulated great interest with its goal to harness patient's own immune system against tumor cells.217 One promising area in immunotherapy is the application of genetically engineered T cells, known as chimeric antigen receptor (CAR) T cells, which allows targeting of tumor associated antigens and could enhance therapy response.218,219 The preparation of functional CAR T cells requires several key steps (**Figure 3**): firstly, the patient's white blood cells are collected and patient’s T cells are isolated via leukapheresis, after which T cells are reengineered and modified with tumor-antigen-specific receptors and co-stimulating molecules; next, a CAR-containing viral vector are transduced into the modified T cells, following amplification of the CAR-expressing T cells before patient infusion. CARs are synthetic receptors that typically contain the following parts: an antibody-derived targeting ectodomain that recognizes tumor antigens, a costimulatory molecule region that could bind to receptors such as CD28, 4-1BB, or CD278,220 and a T-cell signaling domain. After binding to a particular antigen, the CAR can transmit signal and activate the modified T cells. The independence of CAR recognition endows genetically engineered CAR T cells with a fundamental antitumor advantage by avoiding the limitation traditionally conferred by the major histocompatibility complex (MHC).221 However, due to the complexity of the manufacturing process, limited selection of target antigens and insufficient anti-tumor responses to solid tumors, the applicability of this transformative product is highly limited. Over the past few years, flexible gene editing technologies have become significant engineering tools to address these limitations and further improve CAR T designs.

Development of allogeneic CAR T cell therapy would simplify and solve some challenges in the manufacturing process of autologous CAR-T cell therapy.222 The endogenous αβ T-cell receptor (TCR) is responsible for major and minor histocompatibility antigens recognition. By genetically disrupting various parts of the αβ TCR complex and/or human leukocyte antigen (HLA) class I loci of allogeneic T cells, it is possible to create a universal cellular therapy product that confers a wider range of application capability with minimal related adverse effects including graft-versus-host disease (GVHD). In 2012, Torikai et al. used engineered ZFNs to eliminate the expression of α or β chains in endogenous TCR, leading to loss of TCR function in CD19 CAR T-cells.223 These modified T cells did not respond to TCR-specific stimuli, but retained the ability to recognize and target CD19, leading to the generation of universal allogeneic tumor-associated antigen-specific CAR T cells. With the same approach, selective elimination of HLA expression was achieved on CD19-specific T cells and on embryonic stem cells, which raised applicability of this strategy by avoiding the infusion of HLA-disparate immune cells.224 Similar work has also been done by Poirot, et al. using TALEN-mediated editing in 2015. By application of TALEN-mediated gene editing, the expression of αβ TCR was inactivated, eliminating the possibility of T cell responses to allogeneic antigens and GVHD.74 The beneficial role of TCR-depleted CD19 CAR-T cells in evading GVHD has recently been validated in two infant patients with relapsed refractory CD19+ B cell acute lymphoblastic leukemia, leading to successful molecular remissions within 4 weeks.225 In addition, the target of lymphocytic depleting monoclonal antibody alemtuzumab, CD52, a human glycoprotein found on the surface of lymphocytes, was simultaneously disrupted by TALEN to eliminate the potential of any remaining alloreactive T cells and promote engraftment of cellular therapies. As a proof of application of this platform, TCR/CD52-deficient CAR T-cells were administered concurrently with alemtuzumab and demonstrated antitumor activity in lymphoma murine model similar to unmodified anti-CD19 CAR T-cells, with resistance to alemtuzumab destruction.226

The widespread use of gene editing techniques based on ZFN- and TALEN has been hampered by the requirement to design specific nuclease pairs for each new gene target. The development of CRISPR/Cas9 system has successfully promoted multiple gene editing in CAR T cells in a faster and easier way. With this technology, Liu, X. et al. efficiently generated CAR-T cells in which two (TRAC and B2M) or three genes (TRAC, B2M and PD-1) were simultaneously disrupted and tested their anti-tumor function in vitro and in vivo.227 To target the first exon of TRAC and B2M, they designed four sgRNAs, respectively. To target the first exon of PD-1, two sgRNAs were designed and one published sgRNA was tested. Finally, double-knockout of (B2M and TRAC) T cells were induced with high efficiency, yet in triple-knockout (B2M, TRAC and PD-1) T cells, only 64.7% of the clones of the PD-1 PCR products were mutants, which implies that PD-1 expression might be downregulated during T cell expansion. More importantly, the CRISPR/Cas9-mediated multiplex gene edited CAR-T cells maintained CD19-specific antitumor function in a lymphoma xenograft mouse model, suggesting that they are promising reagents for cancer treatment. In another interesting study,228 efficient double knockout of endogenous TCR and HLA class I molecule was achieved by a one-shot CRISPR protocol that incorporated multiple gRNAs into a CAR lentiviral vector to generate allogeneic universal CAR T cells. In this study, the CRISPR/Cas9 mediated simultaneous knockout of 4 loci of T-cell surface receptors PD-1 and CTLA-4 and also successfully generated allogeneic universal T cells. More recently, CRISPR/Cas9 mediated generation of CAR T cells that specifically disrupt inhibitory immune receptors such as T cell membrane protein-3 (TIM-3),229 adenosine 2a receptor (A2aR)230 and lymphocyte-activation protein 3 (LAG-3),231 have shown better percentage of complete remission in xenograft mouse models by increasing the secretion of anti-tumor related cytokines (such as IFN-g, GM-CSF and MIP-1b). These factors may be involved in CAR T cell exhaustion and acute myeloid leukemia (AML) dysfunction, as the combination of checkpoint inhibitors with CAR T cells may result in enhanced antitumor efficacy of AML and other hematologic malignancies.

All together, these results suggest that genome editing could serve as a good platform for generating “universal” CAR T cells and can be applied to large-scale production of healthy “off-the-shelf” T cells against multiple targets.

* 1. **Screening for functional genes**

The concept of precision medicine has led to the development of many targeted drugs for the treatment of different diseases. For example, the targeted drugs designed for the known carcinogenic sites will specifically bind to the carcinogenic components (gene fragment or protein) and induce apoptosis of tumor cells without affecting normal tissue cells. However, one obvious drawback of this molecular targeting therapy is that only those who exhibit a certain mutation or gene expression alteration will respond to the targeted drug; On the other hand, drug resistance persists. Based on the CRISPR/Cas9 technology, scientists have established mammalian genome-wide mutation libraries or libraries of gene mutations associated with certain functions, which is related to screening phenotypes through functional screening and subsequent PCR amplification and deep sequencing analysis. The entire process, called the CRISPR/Cas9 gRNA library screening technology.232,233 The gRNA library is an ideal tool for drug screening or targeted screening of specific pathways. The establishment of gRNA libraries will play an important role in functional gene screening, disease mechanism research and drug development. Functional genome screening using the CRISPR system could reveal changes in gene expression after cancer drug therapy and help to investigate drug-gene interactions by adding small molecules as perturbations, thereby identifying novel targets for precise treatment and providing insights into disease development.234,235

One of the chief goals of pooled CRISPR/Cas9 unbiased screening in cancer research is to identify genotype-specific vulnerabilities and acute myeloid leukemia (AML) was the first disease to be systematically applied with this technology.236 Using this platform, the authors found several well-known targets potential for AML therapies, including BCL2, BRD4, MEN1 and DOT1L, by studying five commonly used AML cell lines and two solid tumor cell lines as controls. Since then, large-scale CRISPR/Cas9 screening has been performed to systematically discover essential genes in many cancer cell lines237,238, and about 1500 essential genes have been identified, which is five times higher than the number of genes previously detected by shRNA screening.239 Another successful example involved the use of CRISPR/Cas9 mediated loss-of-function screening was to identify cancer metastasis-related genes.240 In the study, a non-metastatic lung cancer cell line was infected with the mouse genome-scale CRISPR knockout (mGeCKO) sgRNA library and subcutaneously transplanted into immunocompromised mice. After six weeks, enriched sgRNA sequencing was performed in mice with lung cancer metastasis, and several candidate genes related to lung metastasis were identified and verified, including the already known genes PTEN 241, miR-345,242 miR-152,243 and several new genes Fga, Trim72 and Nf2. Using a CRISPR-based strategy, another loss-of-function screening identified four candidate hepatocellular carcinoma (HCC) suppressor genes that had not previously been associated with HCC (Nf1, Plxnb1, Flrt2, and B9d1). The authors also found that these suppressor genes were closely related to the RAS signaling pathway through the intervention of small molecule inhibitors.244 A CRISPR-based double knockout (CDKO) system has also been developed in K562 leukemia cells. The system uses dual sgRNA libraries to screen for combinatorial genes and identify pairs of synthetic lethal drug targets.245 Recent landmark studies have demonstrated the power of CRISPR/Cas9 to discover long non-coding (lncRNA) loci. These studies applied CRISPR-interference (CRISPRi)- or CRISPR-activation (CRISPRa)-based library to screen for functional lncRNA loci that could modify cell proliferation246,247 and drug resistance235,248. Generally, a comprehensive sgRNA library was designed to target the initiation site of lncRNA transcription and then the library was transduced into different cell lines. Then through sequence analysis, hundreds to thousands of lncRNA promoting cell growth and drug resistance could be identified.

Depending on each mutation’s individual effect, simultaneous mutation of two genes can produce an unexpected phenotype that determines the potential functional relationship between genes.249 This phenomenon, known as genetic interaction, has implications for the development of cancer therapeutic, for example, in cancers with loss-of-function mutations in BRCA1 or BRCA2, an inhibitor of PARP1/2 (eg. olaparib) could result in cell killing by simultaneously disrupting both genes.250 The CRISPR/Cas9 system provides an effective strategy for identifying synergistic gene interactions to gain insights into the response of cancer to chemotherapy. A CRISPR-based double knockout system combined with deep sequencing, phenotypic measurement and genetic analysis has identified interactions between the synergistic drug targets in K562 leukemia cells, such as BCL2L1 and MCL1.245 Similarly, the double-knockout screening method was used to detect 73 tumor genes in pairs and found synthetic lethal interactions of many known (e.g., BRCA-PARP) and unknown genes, approximately 75% of which could be replicated using combinatorial drugs.251 Combining pooled CRISPR screening with a perturbed drug could identify genes that synergize or confer resistance to the agent.252 In one of the first pooled CRISPR screens, the BRAF inhibitor vemurafinib was used to treat a genome-scale knockout library of melanoma cells and recovered genes conferring resistance to the drug.253 Similar to the CDKO system, another simple and effective strategy for analyzing the function of combinatorial genes is CombiGEM-CRISPR (combinatorial genetics en masse-CRISPR).232 It combines two pooled sgRNA libraries in one vector, and some genetic hits (such as KDM6B and BRD4) were discovered by this method. Disrupting these genes with CombiGEM system demonstrated a stronger synergistic effect on the proliferation of tumor cells compared to the already reported small molecule inhibitors. Likewise, a series of CRISPR-based screening techniques have been performed to identify genes that regulate cellular response to specific drugs, such as TRAIL,254 ATR255 or Ras256 pathway inhibitors. Of note, a in vivo screening based on CRISPR/Cas9 has identified protein tyrosine phosphatase non-receptor type 2 (PTPN2) as a novel target for cancer immunotherapy.257 In the future, this innovative approach could also be used to develop personalized cancer therapies based on genotype-specific targets.258

* 1. **Gene diagnosis tools**

Cancer predisposition genes describe genes in which germline mutations result in increased risk of cancer.259 Identifying such sensitive genes through genetic diagnosis is critical for cancer prevention. However, low-frequency mutations are not easily identified by sequencing, a CRISPR-based diagnostic system referred to as SHERLOCK (specific high sensitivity enzymatic reporter UnLOCKing) has been established to solve this problem.260 Technically, the system consists of two important elements, the RNA-guided endonuclease Cas13a (another Cas family member), and the reporter signal. Cas13a exists as a key factor and effectively induces trans-cleavage of non-specific single-stranded DNA (ssDNA). The reporter signal is released after RNA cleavage. This approach appeared to be a highly sensitive detection method when used to detect two cancer mutants, BRAF V600E and EGFR L858R.57 Another system called DETECTR (DNA endonuclease-targeted CRISPR trans reporter) has also been developed.261 Cas12a acts like Cas13a in this system, and another enzyme, recombinase polymerase amplification (RPA), is used as a detection tool to screen for viral infections in cancer and to amplify microsamples. The system seemed to be a fast and inexpensive method for detecting HPV 16/18 in lung carcinomas.262 In the study of breast cancer, the CRISPR nuclease-dead Cas9 (dCas9) system was fused to a DNA methyltransferase effector and infected healthy breast cells by lentivirus. Through this technology, researchers have discovered that the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene was a key driver in carcinogenesis, which led to abnormally rapid cell division and might become an early diagnostic marker for breast cancer.263 Additionally, CRISPR/Cas9 gene editing as well as overexpression experiments has also confirmed that the BRCA1-delta11q optional splice isoform as a primary factor in PARPi and cisplatin treatment resistance of breast cancer.264

1. **Application of gene editing in clinical trials**

Genome editing, as an attractive and challenging therapeutic approach, can correct or eliminate mutations that lead to the development of cancer and other genetically-driven diseases. So far, ex vivo genome editing has been the most widely used, that is, genetic engineering of cells in vitro and then the modified cells are re-engrafted back to patients. In recent years, teams represented by China and the United States have conducted a series of clinical trials of gene editing, such as producing more effective CAR T cells for the treatment of cancer and knockout the erythroid specific enhancer of BCL11A to up-regulate gamma globulin in autologous erythroid HSCs as a potential therapy for sickle cell disease and β-thalassemia (**Table 2**).

**5.1 Anticancer clinical trials**

The gene editing clinical trial using ZFNs product GRm13Z40-2 for the treatment of stage III or IV malignant glioma patients (NCT01082926) was launched in 2010. The ZFNs-mediated GRm13Z40-2, an allogeneic CD8+ cytolitic T cell line genetically modified to express the glucocorticoid-resistant IL13-zetakine, was delivered to tumor cells by intra-tumoral injection. In another phase I clinical trial (NCT02800369), ZFNs agents (ZFN-603 and ZFN-758) were transfected into HPV-infected cervical epithelial cells to determine whether these agents would be able to block the malignant progression of cervical intraepithelial neoplasia and reduce the incidence of cervical cancer. Up to now, this study has finished the data collection phase. Only two studies using TALENs in CAR T cells have been reported. One study (NCT02808442) developed a portfolio of allogeneic, universal CAR T cells (UCART19) that target relapsed or refractory CD19-positive B-acute lymphoblastic leukemia. In this study, alloreactivity and alemtuzumab sensitivity were eliminated by disrupting the loci encoding the TRAC and CD52. Similar concept is used to generate allogeneic TALEN-edited CAR T cells that target CD123 (UCART123) in acute myeloid leukemias and blastic plasmacytoid dendritic cell neoplasms (NCT03190278).

Due to the simple design process and the ability to carry out multi-editing at one time, CRISPR/Cas9 system has become an important tool in the development of cancer therapy. Until now, eleven clinical trials have been carried out to assess the effectiveness of CRISPR system in cancer therapy, 7 of which are immunotherapies that target PD-1protein expression. The first clinical trial using the revolutionary CRISPR/Cas9 technique for cancer treatment recruited its first patient in West China Hospital, Sichuan University in 2016.265 In this non-randomized, open-label phase I study (NCT02793856), the safety of ex vivo engineered PD-1 knockout T cells has been evaluated in the treatment for metastatic non-small cell lung cancer with progression after all standard treatments. In this trial, PD-1 expression was disabled by CRISPR/Cas9 in peripheral blood lymphocytes harvested from the enrolled patients. The edited lymphocytes were isolated, expanded and subsequently reinfused into the patients. Ongoing clinical trials apply the same concept of PD-1 knockout autologous T cells to treat other cancer types, including prostate cancer (NCT02867345), esophageal cancer (NCT03081715) and renal cell cancer (NCT02867332). These trials can be considered as first proof-of-concept studies to apply the in vitro CRISPR/Cas9 gene knockout technique in cancer therapy. There are now studies combining PD-1 knockout with other targeted editing in therapy development, which may lead to improved efficacy for clinical application. One example is the addition of PD-1 knockout to Eppstein-Barr virus (EBV)-specific autologous T-cells for the treatment of EBV-positive cancers, currently in phase I/II clinical trials (NCT03044743).

The elimination of endogenous TCR and PD-1 by CRISPR might enhance tumor rejection activity. Recently, the Recombinant DNA Advisory Committee (RAC) of US National Institutes of Health (NIH) approved a clinical trial to be piloted at the University of Pennsylvania. In this trial, PD-1 and the endogenous TCR will be abolished by CRISPR/Cas9 in HLA-A\*0201 restricted NY-ESO-1 TCR redirected autologous T cells. Such redirected engineered T cells will be applied to a variety of cancer types, including relapsed refractory multiple myeloma, melanoma, synovial sarcoma, and myxoid/round cell liposarcoma (NCT03399448).

The use of CRISPR/Cas9 technology to generate CAR T cells to attack malignant cells has become a research hotspot in clinical trials. A clinical phase 1/2 trial (NCT 03166878) was conducted to evaluate the safety and tolerance of patients with recurrent or refractory CD19+ leukemia and lymphoma to several doses of universal CD19-specific CAR-T cells (UCART 019). In this study, UCART019 cells were obtained by combing lentiviral delivery of CAR receptors and CRISPR RNA electroporation to simultaneously disrupt endogenous TCR and B2M genes. These cells derived from one or more healthy unrelated donors but might help to avoid the graft-versus-host-disease (GVHD) and reduce host-mediated immunity, thereby providing patients with anti-leukemic effects in a relatively safe condition. Unfortunately, a small number of patients relapsed due to the lack of CD19 expression in tumor cells. Therefore, another clinical trial (NCT03398967) that is more applicable for a wide range of patients focused on allogenic CRISPR-edited bispecific CD19+CD20+ or CD19+CD22+ CAR T cells, which could recognize and kill the CD19-negative malignant cells through recognition of CD20 or CD22. In another study, a new clinical trial (NCT03057912) has proposed to evaluate the safety and efficacy of combination genome editing of TALENs and CRISPR/Cas9 by targeting HPV16 and HPV18 E6/E7 DNA in the treatment of HPV-associated cervical intraepithelial neoplasia. In this trial, CAR T cells edited by both techniques were administered twice a week for four weeks to disrupt target gene expression and promised to reduce off-target effects.

The mutation rate of the neurofibromatosis type 1 (NF1) gene is one of the highest in the human genome which is likely to cause various benign or malignant tumors.266 In one trial (NCT03332030), CRISPR/Cas9 technology has been designed to screen and identify NF1 specific drugs. First, an human iPSC library was established from NF1 patients with good phenotypic characteristics, and different cell lines (NF1+/+, NF1+/− and NF1−/−) were developed using CRISPR/Cas9. Then potential therapeutic agents could be identified by examining the reversal or remission phenotypes after specific drug use. Although results from clinical trials in genome editing appear to be promising, more work needs to be done to ensure the safety and effectiveness of this tool in treating human cancers.

**5.2 Antiviral clinical trials**

CCR5 acts as a major coreceptor in the early stage of HIV infection, and CXCR4 plays an important role as an auxiliary receptor when establishing stable infections. Treatment strategy targeting both co-receptors may avoid protection failure because co-receptors usage of HIV infection can be switched between CCR5 and CXCR4.267 The production of engineered immune cells resistant to HIV infection or replication is the primary strategy for genome editing-based HIV treatment. The most common method involves two steps: modifying the cells (CD4+ T cells and CD34+ hematopoietic stem/progenitor cells) in vitro and then reinfusing the modified cells into patients.268,269 Several clinical trials involving CD4+ T cells modification in the context of HIV infection have already been tested. The first approved genome editing trial involving the treatment of HIV with ZFNs (NCT00842634) began in 2009 to evaluate the safety and anti-HIV effects of modified autologous CD4+T cells in HIV-1 infected patients. The ZFNs were delivered ex vivo to autologous CD4+ T cells by adenoviral vectors for CCR5 gene knockout and each participant received a single infusion of 5 to 10 billion ZFN-modified CD4+ T cells. The clinical outcome was published in 2014270 and indicated that CCR5-knockout cells were protected from CCR5-tropic HIV infection and the infusions of genetically engineered T cells into patients were well tolerated, with only 1 patient presented with minor infusion-related adverse events. Since the preliminary demonstration of clinical safety, the main purpose of follow-up trials have been to further optimize the therapeutic effect of gene-edited T cells. Sangamo and/or the University of Pennsylvania tried to improve engraftment of the infused T cells by increasing the number of genetically modified CD4+ T cells, clearing nonmyeloablative lymphocyte, using multiple infusions of cells and switching from adenoviral vector delivery to mRNA electroporation. Although recent advances in ZFN-modified CD4+ T cell infusion have provided some evidences for the safety and low off-target rate of this therapy, a long-term evaluation is still needed. Another study provided proof for the safety of the permanent gene disruption of CCR5 in autologous CD34+ hematopoietic stem/progenitor cells (HSPCs) with ZFN ex vivo (NCT02500849). The main advantage of using HPSCs over T cells is that we will be able to obtain a large number of cells subsets that are protected from HIV infection, which are differentiated by the genetically edited CD34+ population. A recently reported article showed that Chinese scientists have established a CRISPR/Cas9-modified CCR5 gene editing system for adult HPSCs to achieve long-term and stable hematopoietic system reconstruction after infusion of modified CD34+ cells into patients with HIV-1 infection and ALL (NCT03164135).271 This study preliminarily proved the feasibility and safety of gene editing adult HPSCs transplantation in human body, and would promote the development of gene editing technology in clinical application. Because HSPCs-based gene therapy is often confined by ex vivo culture techniques and difficulties in HPSCs expansion, there is also interest in modifying patient-specific iPSCs and reprogramming them to HSPCs.272 (Clinical trials involving HPV and EBV infection are described in the Anticancer clinical trial section).

**5.3 Clinical trial of hematological diseases**

Up to date, ZFN and CRSPR/Cas9 have been applied in five clinical gene therapeutic trials pertaining to hematological diseases, including hemophilia B, β-thalassemia, and sickle cell disease.

Hemophilia B is a recessive, X-linked hemorrhagic disease represented by lack of expression of coagulation factor IX (F IX).273 In November 2016, Sangamo Therapeutics Inc. initiated a phase I clinical trial (NCT02695160) with expected 12 participants using SB-FIX, which is an adeno-associated virus-delivered ZFN, designed to intravenously delivered to the subject’s own hepatocytes to insert a corrective FIX transgene into albumin locus; thus they aim to achieve permanent FIX clotting factor production in the liver of severe hemophilia B patients. This ascending dose phase I study attempts to assess the safety and tolerability of SB-FIX in treating hemophilia B patients, expected to complete in January 2021. Abnormality in the β-globin gene (HBB) can reduce the synthesis of β-globin chains in hemoglobin, causing β-Thalassemia.274 In January 2019, Allife Medical Science and Technology Co., Ltd. Started a 12-subject early phase I trial, where they have applied CRISPR/Cas9 to correct HBB gene in vitro in patient-specific induced hematopoietic stem cells (iHSCs), and intravenously transfuse the edited cells back to the HBB mutated β-thalassemia subjects. This trial is expected to complete in 2021. BCL11A, a key modifier in hemoglobin disorders, characterized by repressing fetal hemoglobin (HbF), is associated with clinical severity of β-thalassemia and sickle cell disease.275 Hence, gene therapy targeting BCL11A to treat the two diseases above have been tested in trials. Until now, there have been three trials tried to suppress BCL11A gene in autologous CD34+ hematopoietic stem/progenitor cells in vitro, and then transfuse the modified cells intravenously back to the subjects, all initiated in 2018 and expected to complete in 2020-2022. Sangamo Therapeutics Inc. has lead the first trial NCT03432364, a single-dose Phase1/2 study with 6 subjects of transfusion-dependent β-thalassemia (TDT). ZFN has been applied to generate the gene-edited therapeutic cell ST-400; its safety, tolerability, and effects on HbF are to be evaluated, transfusion requirements to be assessed. Another single-dose Phase 1/2 study trial (NCT03655678) with up to 45 subjects, focusing on transfusion-dependent β-thalassemia (TDT), has been initiated by Vertex Pharmaceuticals Inc.; they utilized CRISPR/Cas9-Modified cell CTX001, aiming to test its safety and efficacy. With similar study design and start/complete time, Vertex Pharmaceuticals Inc. have also tested CTX001 in severe sickle cell disease (NCT03745287).

**5.4 Clinical trials of hereditary eye diseases**

Gene augmentation are successfully employed for the treatment of inherited retinal diseases, and a large number of clinical trials of gene augmentation are underway for LCA, choroideremia, achromatopsia, X-linked retinoschisis and RP.276 Until now, there has been only one clinical trial of gene editing in LCA10. Recently, a clinical study (NCT03872479) was initiated by Editas and Allergan to evaluate the safety, tolerability and efficacy of single dose AGN-151587 (EDIT-101), an AAV vector containing 3 components: including a S. aureus Cas9 and two gRNAs--gRNA-323 and gRNA-64. AGN-151587 could eliminate the mutation of c.2991+1655A>G in intron 26 of the CEP290 gene to treat LCA10. Although clinical trials on gene editing for the ophthalmic diseases have just begun, the unique qualities of eyes, such as easy accessibility and relative immune-privileged status, make CRISPR-Cas become a promising and available strategy for ophthalmic disease treatment in the near future.164,276

1. **Challenges in therapeutic targeting**

In addition to the many benefits of genome editing, there are some technical challenges in translating these treatments to clinical disease therapy, primarily in terms of accuracy, efficacy and delivery hurdles. To cope with these challenges, scientists will need profound knowledge about molecular nature of cancers, especially heterogeneous solid tumors, as well as carefully designed genome editing platforms in preclinical studies.

**6.1 Increasing specificity of gene correction.**

The accuracy of gene editing technology is defined by the ability to edit desired locus of interest within the genome. While inevitably, mutations in undesired genomic loci, namely off-target effects, are rather pernicious, as they can lead to potential genomic toxicity, genome instability, disruption of gene function, epigenetic alterations, and even carcinogenesis.16,277,278 Given that therapeutic gene targeting is strongly dependent on the creation of DSBs at specific target sites, assays of paramount importance have been developed to assess the targeting specificities of ZFN, TALEN and Cas9 nucleases, such as in vitro selection libraries,279,280 mismatch-detection nuclease assay,281 newly reported high-throughput profiling,282 next generation sequencing (NGS)283 and whole genome sequencing (WGS).284,285 Thus, the above studies revealed a number of factors that might affect the specificity of gene editing, which can be roughly divided into two categories. First of all, the intrinsic specificity encoded in the Cas9 protein may determine the relative importance of each position that may differ between different sgRNA sequences. Secondly, the specificity also depends on the abundance of effective nuclease complexes relative to the target concentration.

Compared to ZFNs and TALENs, CRISPR/Cas9 may present higher potential for off-target effects in human cells.278 As previously noted, there is a tolerance of sequence mismatch when Cas9-sgRNA binds to the target DNA: both identical and highly homologous DNA sequences can be cleaved, leading to chromosomal rearrangements or off-target mutations.286,287 With numerous studies demonstrating the presence of its off-target activity, it has become the task with top priority to improve DNA specificity in CRISPR technology.278 Accordingly, several strategies have been exploited to minimize Cas9-mediated off-target effects and increase the cleavage specificity. Both the structure and composition of gRNA can affect the level of off‐target effects.288,289 A related method that has been reported to reduce off-target effects induced by Cas9 is to choose unique target sequences that lack homology to other regions of the genome.290 Meanwhile, the use of truncated and less-active sgRNAs that are shortened at 5ʹ end by two to three nucleotides decreased undesired mutagenesis at some off-target sites because this sgRNA structure has higher sensitivity to mismatches.277,282 Another strategy to reduce the off-target effects is to harness a pair of nCas9 or RNA-guided FokI nucleases to generate paired nicks instead of DSBs, which can significantly avoid off-target cleavage without sacrificing genome editing efficiency.291,292 In addition, the concentration of Cas9-sgRNA delivered to cells should be carefully controlled, as it is another factor that affects off‐target effect.293 However, increasing specificity by reducing the amount of transfected DNA also results in reduced cleavage at the target. Therefore, a balance between on-target cleavage efficiency and off-target effects must be considered. Most recently, two different variants of monomeric Streptococcus pyogenes have been engineered to form a SpCas9 that exhibits improved genome-wide specificities. Slaymaker et al. described an enhanced SpCas9 that contains alanine substitutions at three positions and predicted the interaction of this variant with a non-target DNA strand.294 In another study, Kleinstiver et al. created the SpCas9-HF1 (high fidelity variant 1) by introducing alanine substitutions at four residues in SpCas9 to disrupt non-specific contacts with the phosphorylated framework of the target DNA strand, which interacts with the gRNA.295 These engineered variants of SpCas9 have been engineered by reducing non-specific interaction of proteins with different DNA strands, dramatically improving genome-wide specificity. They do not alter the target range or size of the DNA that is required to encode the desired Cas9 nuclease and a single gRNA, thus functional mutations could also be combined to further increase specificity.

Alternative delivery methods have also been developed to improve specificity of the editing process. Direct delivery of recombinant Cas9 protein and in vitro transcribed sgRNA either alone or in purified complexes, reduced off-target effects when compared with plasmid transfected delivery systems.296,297 Anti-CRISPR molecules, recently discovered inhibitors for CRISPR systems, may add the precise control of genome editing strategy,298 which are currently tested.299

* 1. **Improving efficiency of nuclease editing**

The efficiency of DSB repair pathways mediated by NHEJ and HDR varies greatly between cell types and cell status; however, in most cases, NHEJ is more active than HDR. It has been observed that NHEJ is active throughout the cell cycle of a variety of cell types, including division and post-mitosis.11,300 In contrast, HDR functions primarily in the S/G2 phase and is therefore largely restricted to actively dividing cells, limiting treatments for precise genomic modification of mitotic cells.301,302 This difference makes treatment of diseases that require genetic correction or gene insertion more challenging than those that require gene inactivation. Since NHEJ-mediated DSB repair can be applied to promote high levels of gene disruption in most cell types, the primary challenge to date has been to improve the efficiency of HDR.

Notably, recent studies have reported novel strategies to upregulate the efficiency of genome editing by inhibiting competing DNA repair pathways, primarily NHEJ-mediated DNA repair. Maruyama et al.303 successfully employed SCR7 to inhibit NHEJ by targeting a key enzyme (DNA ligase IV) in NHEJ pathway, thereby increasing the genome editing efficiency in cell lines and mice by up to 19-fold. In another independent study, Kuhn et al. abolished the NHEJ activity in human and mouse cell lines by gene silencing of several key molecules of the NHEJ repair pathway (KU70, KU80 or DNA ligase IV), leading to increased genome editing efficiency.304 Further, Canny et el. discovered that 53BP1, a genetically encoded inhibitor, increased HDR-dependent genome editing efficiency by up to 5.6-fold through suppressing NHEJ activity in human and mouse cells.305 Interestingly, by application of an HDR enhancer, RS-1, Song et al. achieved multifold improvement on the CRISPR/Cas9- and TALEN-mediated knock-in efficiency both in vitro and in vivo, whereas the NHEJ inhibitor SCR7 has minimal effects.306 Identification of novel small molecule inhibitors against other NHEJ proteins, such as artemis and XRCC4, may further advance current strategies.307,308 An improved CRISPR system, called CRISPR/Cpf1 or CRISPR/Cas12a that employs a smaller and simpler RNA-guided DNA nuclease, could target genomic regions that cannot be targeted by Cas9 and induce multiplex gene perturbation in vitro with frequencies of up to 45%.309 Meanwhile, timed delivery of Cas9-guide RNP (RNA ribonucleoprotein) complexes was used to site-specifically induce DSB and new genetic information, with high efficiency of HDR.310 In addition to the methods already mentioned, further researches aimed at improving HDR efficiency will be necessary to optimize genome editing for a wider range of diseases.

Although CRISPR/Cas9 gene editing system improves the efficiency of gene knockout and site-directed modification (including site-directed mutation and gene insertion), the efficiency of gene site-directed mutation based on homologous recombination mechanism is still low. In order to improve the efficiency of site-directed mutation, the base editors (BEs) system combining CRISPR/Cas9 and cytosine deaminase has been reported one after another.311-313 By using this system, the fusion protein composed of Cas9-cytidine deaminase and uracil glycosaminase inhibitor can be targeted at the desired site complementary to gRNA without double strand DNA fragmentation, and the amino group of pyrimidine (C) at the target site can be removed, so that C becomes uracil (U), and U will be replaced by thymidine (T) with the replication of DNA. Finally, the single base C→T mutation is realized accurately and efficiently, leading to single-base-pair substitutions in eukaryotic cells.314 BEs technique adds an important tool to the research and application of genome editing technology.

**6.3 Optimizing delivery system**

One of the key challenges for future application of gene editing tools will be the development of efficient and secure methods to deliver genetic editing elements, not only to the tumor cells ex vivo, but also to somatic cells in vivo. Delivery methods include viral methods and non-viral physical methods (**Figure 4**). Non-viral physical delivery methods, such as electroporation,315 hydrodynamic injection316 and lipid nanoparticles317 have been widely utilized to deliver ZFNs, TALENs, and CRISPR in different cell lines and animal models. Despite their simplicity and safety, the relatively poor delivery efficacy limits therapeutic applications of those non-viral delivery methods in vivo.318 By contrast, viral vectors (such as retroviruses, lentivirus, adenovirus (AdV) and adeno-associated virus (AAV)) have high delivery efficiency, and some of them have been approved for clinical uses.319,320 To date, viral delivery systems have been the most effective system for delivering plasmid-based nucleic acids to mammalian cells in vitro and in vivo, despite the possibility of introducing unintentional mutations and the existence of safety concerns.321-324 Recent studies have further highlighted other issues affecting delivery efficiency, including the immune risk of host humors and cells to Cas9 proteins,325 as well as the DSB P53 responses related to genome editing.326 Many new viral and non-viral systems have been developed to overcome these problems.

Non-viral delivery systems could extend the range of genome editing therapies by alleviating concerns about the safety and immunogenicity of native cells in vivo. For instance, delivery of plasmid DNA encoding a Cas9-sgRNA complex that targets VEGF using a PEG-PEI-cholesterol lipid polymer could achieve gene knockout approximately 50% in osteosarcoma cells in vitro and in vivo.327 A lipid delivery system containing PEG-poly lactic-co-glycolic acid nanoparticles was used to deliver CRISPR DNA constructed by a CD68 promoter and achieve in vitro and in vivo gene editing of specific macrophages.328 Zuris and colleagues also studied lipid materials as vectors for genome-edited proteins. First, they fused Cas9 and TALEN into anionic GFP proteins to increase negative charges on the surface, and then complexed them with Lipofectamine 2000TM (a commercially cationic lipofection reagent); this novel complex achieved 24% gene knockout of mouse embryonic stem cells in vitro and 13% gene knockout of mouse cochlea hair cells in vivo.317 The gene knockout rate of the complex to mouse embryonic stem cells in vitro was 24%.

According to Finn et al., lipid nanoparticles composed of PEG–lipids exhibited excellent serum stability. When used to deliver Cas9 mRNA and sgRNA targeting mouse transthyretin gene in hepatocytes, they caused a drop in serum protein levels of more than 97%, which lasted for at least 12 months after a single systemic injection.329 Recent work by Cheng and Leong et.al. has demonstrated that delivery of Cas9 and sgRNA plasmids with cationic alpha-helical polypeptides is expected to enhance gene-editing efficiency in vitro and in vivo. With this delivery system, repeated intra-tumoral injections in a HeLa xenograft mouse model resulted in ~67% of targeted genes knockdown, >71% of tumor growth inhibition, and ultimately significantly prolonged survival of tumor-bearing mice.330 Moreover, Cas9 protein and sgRNA complex showed higher efficiency than plasmid-based CRISPR/Cas9 and Cas9 mRNA/sgRNA. For example, recombinant Cas9 proteins and sgRNA has been reported to achieve 16% editing efficiency in vitro through cell-penetrating peptide (CPP),297,331 while delivery of purified Cas9 protein mediated by electroporation increased the editing efficiency to 79%,296,332 because transgenic proteins degraded rapidly and avoided long-lasting effects on the genome.

To improve the specificity and safety of viral-mediated gene-editing delivery, different parts of preexisting viruses can be mixed together creating hybrid virus vectors. The structure of virus can be tweaked by point mutations, or virus can incorporate with small molecules, synthetic polymers and inorganic nanoparticles.333 For example, lentiviral vectors are typically pseudotyped with glycoprotein G from vesicular stomatitis virus (VSV-G), extending the vector tropism to a wide range of host cells.334 By controlling the ratio of assembled wild-type viral capsid to protease activatable subunits, the overall transduction level of protease-activatable viruses (PAVs) increased.335 Using error-prone polymerase chain reaction (EP-PCR), Asuri et al. created a library of AAV capsid genes with point mutations, which resulted in a viral variant that was more efficient in delivering genetic payloads to human stem cells.336 The vector is further enhanced by conjugative delivery to zinc finger nucleases: the induced DSB facilitated HDR repair of the delivered transgene, thereby enabling gene targeting. Another way to further modify or enhance the functional properties of viruses is by incorporating synthetic non-biological components such as polymers and nanoparticles. Hofherr et al.337 attached PEG-5000 to adenoviral vectors to generate adeno-PEG-injected counterparts (Ad-PEG). After intravenous injection to mammalian blood, the PEGylation blunted interactions of adenovirus with platelets and endothelial cells and reduced thrombocytopenia as well as D-dimer formation. In another study, Lee et al. investigated the possibility of conjugating the AAV surface-exposed lysine on the capsid with the activated PEG chains of PEG-2000 to protect the AAV vectors from neutralizing antibodies.338 At a critical conjugation ratio, the particles were moderately protected from serum neutralization by 2.3-fold over the unmodified vectors. These results indicate that certain modifications of viral vectors may have utility to reduce immune responses that are involved in the delivery process, thereby improving their safety for human gene therapy.

Proper selection of different delivery systems and CRISPR/Cas9 types also contributes to the reduction of off-target effect. For instance, the use of minicircle DNA is more efficient and less immunogenic than plasmid DNA per-mass due to the elimination of bacterial expression sequences.339 Co-delivery of Cas9 and EGFR mutation-specific sgRNAs by adenovirus could precisely disrupt the oncogenic mutant allele, showing high specificity.340 Furthermore, non-viral polymers that conjugated to the gold nanoparticle hybridization system have been recognized as a suitable vehicle for delivery of Cas9 RNP complexes plus donor DNA, which could effectively correct the disease phenotypes of muscle cells after intramuscular injection.341

1. **Conclusions** **& future perspective**

The research evidence accumulated to date has demonstrated significant contributions of genome editing systems to exploit therapeutic strategies for various types of human diseases, among which CRISPR/Cas9 system has been especially effective via directly interfering with target gene loci or deriving multifunctional tools. In the future, combination of pooled CRISPR screening and the existing information on the genetic and epigenetic characteristics of cancer cell lines will be able to broadly identify synthetic lethal interactions in the genome, and facilitate the discovery of novel drug targets. The CRISPR/Cas9 platform also provides a new tool to manipulate non-coding regions of the cancer genome, accelerating the functional exploration of aspects that are hitherto poorly characterized. The tremendous advances in the development of engineered nucleases (especially ZFN, TALEN, and CRISPR/Cas9) paved the way for genome editing from theoretical concept into clinical practice. At the end of 2017, Brian Madeux, an American man with Hunter's syndrome, received a bold treatment at the Benioff Children's Hospital at the University of California, San Francisco: delivering ZFN via AAV for in vivo genetic editing to treat his disease. This is the first report in the world to treat genetic diseases through in vivo gene editing, which further demonstrates that gene editing has extremely important clinical application potential for the treatment of genetic diseases. Genome editing technology has also been combined with tumor immunotherapy to provide more updated options for human disease treatment. As one of the most innovative and successful approaches in tumor immunotherapy, CAR-T cell therapy was officially approved into the clinic in 2017. Refractory ALL and CLL patients responded completely to CAR T cell products directly targeting CD19, so the US Food and Drug Administration (FDA) recognized CAR-T cell therapy as a “breakthrough therapy” and approved its treatment for leukemia and lymphoma. The effective response of CAR T therapy in clinical trials of B-cell malignancies has evoked great enthusiasm for the ultimately intelligent treatment, brought hope to cancer patients, and led to the commercialization of CAR-T cells by many pharmaceutical and biotechnology companies. However, the development of CAR T cell therapy is still in its infancy, and the high costs of CAR T cell therapy have made it unaffordable for a large population in the society. Moreover, the commercial potential of this therapy, especially the possibility of becoming an off-the-shelf therapy remains uncertain; besides, its capacity to combat solid tumors remains to be confirmed.

At the same time, gene editing technology has also promoted the development of cell imaging, gene expression regulation, epigenetic modification, therapeutic drug development, functional gene screening, and gene diagnosis. Although the off-target effect in the implementation of gene editing technology still needs further optimization, innovative genome-editing complexes and more specific nanostructured vehicles have improved efficiency and reduced toxicity during the delivery process, bringing genome editing technology closer to the clinic. With deeper exploration into this technology and cooperation of the world scientific community, it is reasonable to believe that genome editing technology has the potential to ultimately elucidate biological mechanisms behind disease development and progression, thus providing novel therapies and finally promoting the development of life sciences.

**Acknowledgements**

This work is supported by the National Natural Science Foundation of China (No. 81602492), the National Key Research and Development Program of China (No. 2016YFA0201402) and by the National Major Scientific and Technological Special Project for “Significant New Drugs Development” (No. 2018ZX09733001).

**Competing interests**

The authors declare that they have no competing interests.

**References**

1 Rothstein, R. J. One-step gene disruption in yeast. *Meth. Enzymol.* **101**, 202-211 (1983).

2 Cornu, T. I., Mussolino, C. & Cathomen, T. Refining strategies to translate genome editing to the clinic. *Nat Med* **23**, 415-423, doi:10.1038/nm.4313 (2017).

3 Ghosh, D., Venkataramani, P., Nandi, S. & Bhattacharjee, S. CRISPR-Cas9 a boon or bane: the bumpy road ahead to cancer therapeutics. *Cancer Cell Int* **19**, 12, doi:10.1186/s12935-019-0726-0 (2019).

4 Gaj, T., Gersbach, C. A. & Barbas, C. F., 3rd. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* **31**, 397-405, doi:10.1016/j.tibtech.2013.04.004 (2013).

5 Rouet, P., Smih, F. & Jasin, M. Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6064-6068 (1994).

6 Kosicki, M., Tomberg, K. & Bradley, A. Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nat Biotechnol* **36**, 765-771, doi:10.1038/nbt.4192 (2018).

7 O'Driscoll, M. & Jeggo, P. A. The role of double-strand break repair - insights from human genetics. *Nat. Rev. Genet.* **7**, 45-54, doi:10.1038/nrg1746 (2006).

8 Kaniecki, K., De Tullio, L. & Greene, E. C. A change of view: homologous recombination at single-molecule resolution. *Nat. Rev. Genet.*, doi:10.1038/nrg.2017.92 (2017).

9 Kim, H. & Kim, J. S. A guide to genome engineering with programmable nucleases. *Nat. Rev. Genet.* **15**, 321-334, doi:10.1038/nrg3686 (2014).

10 Verma, P. & Greenberg, R. A. Noncanonical views of homology-directed DNA repair. *Genes Dev.* **30**, 1138-1154, doi:10.1101/gad.280545.116 (2016).

11 Chang, H. H. Y., Pannunzio, N. R., Adachi, N. & Lieber, M. R. Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nat. Rev. Mol. Cell Biol.*, doi:10.1038/nrm.2017.48 (2017).

12 Lieber, M. R., Gu, J., Lu, H., Shimazaki, N. & Tsai, A. G. Nonhomologous DNA end joining (NHEJ) and chromosomal translocations in humans. *Subcell. Biochem.* **50**, 279-296, doi:10.1007/978-90-481-3471-7\_14 (2010).

13 Delacôte, F. & Lopez, B. S. Importance of the cell cycle phase for the choice of the appropriate DSB repair pathway, for genome stability maintenance: the trans-S double-strand break repair model. *Cell Cycle* **7**, 33-38 (2008).

14 Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S. & Gregory, P. D. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* **11**, 636-646, doi:10.1038/nrg2842 (2010).

15 Silva, G. *et al.* Meganucleases and other tools for targeted genome engineering: perspectives and challenges for gene therapy. *Curr Gene Ther* **11**, 11-27 (2011).

16 Cathomen, T. & Keith Joung, J. Zinc-finger Nucleases: The Next Generation Emerges. *Molecular Therapy* **16**, 1200-1207, doi:10.1038/mt.2008.114 (2008).

17 Boch, J. *et al.* Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* **326**, 1509-1512, doi:10.1126/science.1178811 (2009).

18 Zhang, F. *et al.* Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. *Nat. Biotechnol.* **29**, 149-153, doi:10.1038/nbt.1775 (2011).

19 Al-Attar, S., Westra, E. R., van der Oost, J. & Brouns, S. J. Clustered regularly interspaced short palindromic repeats (CRISPRs): the hallmark of an ingenious antiviral defense mechanism in prokaryotes. *Biol Chem* **392**, 277-289, doi:10.1515/BC.2011.042 (2011).

20 Cox, D. B., Platt, R. J. & Zhang, F. Therapeutic genome editing: prospects and challenges. *Nat Med* **21**, 121-131, doi:10.1038/nm.3793 (2015).

21 Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819-823, doi:10.1126/science.1231143 (2013).

22 Mali, P. *et al.* RNA-guided human genome engineering via Cas9. *Science* **339**, 823-826, doi:10.1126/science.1232033 (2013).

23 Kim, Y. G., Cha, J. & Chandrasegaran, S. Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1156-1160 (1996).

24 Diakun, G. P., Fairall, L. & Klug, A. EXAFS study of the zinc-binding sites in the protein transcription factor IIIA. *Nature* **324**, 698-699, doi:10.1038/324698a0 (1986).

25 Beerli, R. R. & Barbas, C. F. Engineering polydactyl zinc-finger transcription factors. *Nat. Biotechnol.* **20**, 135-141, doi:10.1038/nbt0202-135 (2002).

26 Beerli, R. R., Schopfer, U., Dreier, B. & Barbas, C. F. Chemically regulated zinc finger transcription factors. *J. Biol. Chem.* **275**, 32617-32627, doi:10.1074/jbc.M005108200 (2000).

27 Buck-Koehntop, B. A. *et al.* Molecular basis for recognition of methylated and specific DNA sequences by the zinc finger protein Kaiso. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 15229-15234, doi:10.1073/pnas.1213726109 (2012).

28 Fairall, L., Schwabe, J. W., Chapman, L., Finch, J. T. & Rhodes, D. The crystal structure of a two zinc-finger peptide reveals an extension to the rules for zinc-finger/DNA recognition. *Nature* **366**, 483-487, doi:10.1038/366483a0 (1993).

29 Guo, J., Gaj, T. & Barbas, C. F. Directed evolution of an enhanced and highly efficient FokI cleavage domain for zinc finger nucleases. *J. Mol. Biol.* **400**, 96-107, doi:10.1016/j.jmb.2010.04.060 (2010).

30 Smith, J. *et al.* Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. *Nucleic Acids Res.* **28**, 3361-3369 (2000).

31 Beumer, K. J. *et al.* Efficient gene targeting in Drosophila by direct embryo injection with zinc-finger nucleases. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 19821-19826, doi:10.1073/pnas.0810475105 (2008).

32 Paschon, D. E. *et al.* Diversifying the structure of zinc finger nucleases for high-precision genome editing. *Nat Commun* **10**, 1133, doi:10.1038/s41467-019-08867-x (2019).

33 Bogdanove, A. J., Schornack, S. & Lahaye, T. TAL effectors: finding plant genes for disease and defense. *Curr. Opin. Plant Biol.* **13**, 394-401, doi:10.1016/j.pbi.2010.04.010 (2010).

34 Bogdanove, A. J. & Voytas, D. F. TAL effectors: customizable proteins for DNA targeting. *Science* **333**, 1843-1846, doi:10.1126/science.1204094 (2011).

35 Moscou, M. J. & Bogdanove, A. J. A simple cipher governs DNA recognition by TAL effectors. *Science* **326**, 1501, doi:10.1126/science.1178817 (2009).

36 Li, T. *et al.* TAL nucleases (TALNs): hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain. *Nucleic Acids Res.* **39**, 359-372, doi:10.1093/nar/gkq704 (2011).

37 Mussolino, C. *et al.* A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Res.* **39**, 9283-9293, doi:10.1093/nar/gkr597 (2011).

38 Mercer, A. C., Gaj, T., Fuller, R. P. & Barbas, C. F. Chimeric TALE recombinases with programmable DNA sequence specificity. *Nucleic Acids Res.* **40**, 11163-11172, doi:10.1093/nar/gks875 (2012).

39 Cermak, T. *et al.* Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res.* **39**, e82, doi:10.1093/nar/gkr218 (2011).

40 Reyon, D. *et al.* FLASH assembly of TALENs for high-throughput genome editing. *Nat. Biotechnol.* **30**, 460-465, doi:10.1038/nbt.2170 (2012).

41 Briggs, A. W. *et al.* Iterative capped assembly: rapid and scalable synthesis of repeat-module DNA such as TAL effectors from individual monomers. *Nucleic Acids Res.* **40**, e117, doi:10.1093/nar/gks624 (2012).

42 Schmid-Burgk, J. L., Schmidt, T., Kaiser, V., Höning, K. & Hornung, V. A ligation-independent cloning technique for high-throughput assembly of transcription activator–like effector genes. *Nat. Biotechnol.* **31**, 76-81, doi:10.1038/nbt.2460 (2013).

43 Ishino, Y., Shinagawa, H., Makino, K., Amemura, M. & Nakata, A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *J. Bacteriol.* **169**, 5429-5433 (1987).

44 Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821, doi:10.1126/science.1225829 (2012).

45 Bolotin, A., Quinquis, B., Sorokin, A. & Ehrlich, S. D. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology (Reading, Engl.)* **151**, 2551-2561, doi:10.1099/mic.0.28048-0 (2005).

46 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 (Reading, Engl.)* **151**, 653-663, doi:10.1099/mic.0.27437-0 (2005).

47 Makarova, K. S. *et al.* An updated evolutionary classification of CRISPR-Cas systems. *Nat. Rev. Microbiol.* **13**, 722-736, doi:10.1038/nrmicro3569 (2015).

48 Makarova, K. S. *et al.* Evolution and classification of the CRISPR-Cas systems. *Nat. Rev. Microbiol.* **9**, 467-477, doi:10.1038/nrmicro2577 (2011).

49 Jiang, W., Bikard, D., Cox, D., Zhang, F. & Marraffini, L. A. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat. Biotechnol.* **31**, 233-239, doi:10.1038/nbt.2508 (2013).

50 Sternberg, S. H., Redding, S., Jinek, M., Greene, E. C. & Doudna, J. A. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* **507**, 62-67, doi:10.1038/nature13011 (2014).

51 Deveau, H. *et al.* Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. *J. Bacteriol.* **190**, 1390-1400, doi:10.1128/jb.01412-07 (2008).

52 Gasiunas, G., Barrangou, R., Horvath, P. & Siksnys, V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **109**, E2579-2586, doi:10.1073/pnas.1208507109 (2012).

53 Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* **8**, 2281-2308, doi:10.1038/nprot.2013.143 (2013).

54 Doudna, J. A. & Charpentier, E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* **346**, 1258096, doi:10.1126/science.1258096 (2014).

55 Liu, C., Zhang, L., Liu, H. & Cheng, K. Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications. *J Control Release*, doi:10.1016/j.jconrel.2017.09.012 (2017).

56 Biagioni, A. *et al.* Delivery systems of CRISPR/Cas9-based cancer gene therapy. *J Biol Eng* **12**, 33, doi:10.1186/s13036-018-0127-2 (2018).

57 Abudayyeh, O. O. *et al.* RNA targeting with CRISPR-Cas13. *Nature*, doi:10.1038/nature24049 (2017).

58 Merkle, T. *et al.* Precise RNA editing by recruiting endogenous ADARs with antisense oligonucleotides. *Nat. Biotechnol.*, doi:10.1038/s41587-019-0013-6 (2019).

59 Tucker, B. A. *et al.* Transplantation of adult mouse iPS cell-derived photoreceptor precursors restores retinal structure and function in degenerative mice. *PloS one* **6**, e18992, doi:10.1371/journal.pone.0018992 (2011).

60 Homma, K. *et al.* Developing rods transplanted into the degenerating retina of Crx-knockout mice exhibit neural activity similar to native photoreceptors. *Stem cells (Dayton, Ohio)* **31**, 1149-1159, doi:10.1002/stem.1372 (2013).

61 Cai, B. *et al.* Application of CRISPR/Cas9 technologies combined with iPSCs in the study and treatment of retinal degenerative diseases. *Hum Genet* **137**, 679-688, doi:10.1007/s00439-018-1933-9 (2018).

62 Vogelstein, B. *et al.* Cancer genome landscapes. *Science* **339**, 1546-1558, doi:10.1126/science.1235122 (2013).

63 Choo, Y., Sánchez-García, I. & Klug, A. In vivo repression by a site-specific DNA-binding protein designed against an oncogenic sequence. *Nature* **372**, 642-645, doi:10.1038/372642a0 (1994).

64 Do, T. U., Ho, B., Shih, S. J. & Vaughan, A. Zinc Finger Nuclease induced DNA double stranded breaks and rearrangements in MLL. *Mutat Res* **740**, 34-42, doi:10.1016/j.mrfmmm.2012.12.006 (2012).

65 Provasi, E. *et al.* Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. *Nat Med* **18**, 807-815, doi:10.1038/nm.2700 (2012).

66 Tanaka, A. *et al.* A novel therapeutic molecule against HTLV-1 infection targeting provirus. *Leukemia* **27**, 1621-1627, doi:10.1038/leu.2013.46 (2013).

67 Huang, N. *et al.* Induction of apoptosis in imatinib sensitive and resistant chronic myeloid leukemia cells by efficient disruption of bcr-abl oncogene with zinc finger nucleases. *J. Exp. Clin. Cancer Res.* **37**, 62, doi:10.1186/s13046-018-0732-4 (2018).

68 Piganeau, M. *et al.* Cancer translocations in human cells induced by zinc finger and TALE nucleases. *Genome Res* **23**, 1182-1193, doi:10.1101/gr.147314.112 (2013).

69 Puria, R., Sahi, S. & Nain, V. HER2+ breast cancer therapy: by CPP-ZFN mediated targeting of mTOR? *Technol. Cancer Res. Treat.* **11**, 175-180 (2012).

70 Herrmann, F. *et al.* p53 Gene repair with zinc finger nucleases optimised by yeast 1-hybrid and validated by Solexa sequencing. *PLoS One* **6**, e20913, doi:10.1371/journal.pone.0020913 (2011).

71 Reik, A., Yuanyue Zhou, Matthew C. Mendel, D. E. P., 1 DiGiusto,2 & Jensen. Zinc Finger Nucleases Targeting the Glucocorticoid Receptor Allow IL-13 Zetakine Transgenic CTLs To Kill Glioblastoma Cells In Vivo in the Presence of Immunosuppressing Glucocorticoids. *Molecular Therapy* **16**, S13-S14, doi:10.1016/s1525-0016(16)39437-0 (2008).

72 Marchiq, I., Le Floch, R., Roux, D., Simon, M. P. & Pouyssegur, J. Genetic disruption of lactate/H+ symporters (MCTs) and their subunit CD147/BASIGIN sensitizes glycolytic tumor cells to phenformin. *Cancer Res* **75**, 171-180, doi:10.1158/0008-5472.CAN-14-2260 (2015).

73 Miller, J. C. *et al.* A TALE nuclease architecture for efficient genome editing. *Nat Biotechnol* **29**, 143-148, doi:10.1038/nbt.1755 (2011).

74 Poirot, L. *et al.* Multiplex genome edited T-cell manufacturing platform for "off-the-shelf" adoptive T-cell immunotherapies. *Cancer Res.*, doi:10.1158/0008-5472.Can-14-3321 (2015).

75 Wang, J. *et al.* TALENs-mediated gene disruption of FLT3 in leukemia cells: Using genome-editing approach for exploring the molecular basis of gene abnormality. *Sci Rep* **5**, 18454, doi:10.1038/srep18454 (2015).

76 Nyquist, M. D. *et al.* TALEN-engineered AR gene rearrangements reveal endocrine uncoupling of androgen receptor in prostate cancer. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 17492-17497, doi:10.1073/pnas.1308587110 (2013).

77 Cai, Y. *et al.* Loss of Chromosome 8p Governs Tumor Progression and Drug Response by Altering Lipid Metabolism. *Cancer Cell* **29**, 751-766, doi:10.1016/j.ccell.2016.04.003 (2016).

78 Xiao, L. *et al.* LRH-1 drives hepatocellular carcinoma partially through induction of c-myc and cyclin E1, and suppression of p21. *Cancer Manag Res* **10**, 2389-2400, doi:10.2147/CMAR.S162887 (2018).

79 Zhan, T., Rindtorff, N., Betge, J., Ebert, M. P. & Boutros, M. CRISPR/Cas9 for cancer research and therapy. *Semin Cancer Biol* **55**, 106-119, doi:10.1016/j.semcancer.2018.04.001 (2019).

80 Heckl, D. *et al.* Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing. *Nat. Biotechnol.*, doi:10.1038/nbt.2951 (2014).

81 Sánchez-Rivera, F. J. & Jacks, T. Applications of the CRISPR-Cas9 system in cancer biology. *Nat. Rev. Cancer* **15**, 387-395, doi:10.1038/nrc3950 (2015).

82 Sayin, V. I. & Papagiannakopoulos, T. Application of CRISPR-mediated genome engineering in cancer research. *Cancer Lett.*, doi:10.1016/j.canlet.2016.03.029 (2016).

83 Matano, M. *et al.* Modeling colorectal cancer using CRISPR-Cas9-mediated engineering of human intestinal organoids. *Nat. Med.* **21**, 256-262, doi:10.1038/nm.3802 (2015).

84 Roper, J. *et al.* Colonoscopy-based colorectal cancer modeling in mice with CRISPR-Cas9 genome editing and organoid transplantation. *Nat Protoc* **13**, 217-234, doi:10.1038/nprot.2017.136 (2018).

85 Li, A. H. *et al.* Analysis of loss-of-function variants and 20 risk factor phenotypes in 8,554 individuals identifies loci influencing chronic disease. *Nat. Genet.* **47**, 640-642, doi:10.1038/ng.3270 (2015).

86 Abrahimi, P. *et al.* Efficient Gene Disruption in Cultured Primary Human Endothelial Cells by CRISPR/Cas9. *Circ. Res.*, doi:10.1161/circresaha.117.306290 (2015).

87 Carroll, K. J. *et al.* A mouse model for adult cardiac-specific gene deletion with CRISPR/Cas9. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 338-343, doi:10.1073/pnas.1523918113 (2016).

88 Willer, C. J. *et al.* Newly identified loci that influence lipid concentrations and risk of coronary artery disease. *Nat. Genet.* **40**, 161-169, doi:10.1038/ng.76 (2008).

89 Gifford, C. A. *et al.* Oligogenic inheritance of a human heart disease involving a genetic modifier. *Science* **364**, 865-870, doi:10.1126/science.aat5056 (2019).

90 Yang, D. *et al.* Generation of PPARγ mono-allelic knockout pigs via zinc-finger nucleases and nuclear transfer cloning. *Cell Res.* **21**, 979-982, doi:10.1038/cr.2011.70 (2011).

91 Umeyama, K. *et al.* Generation of heterozygous fibrillin-1 mutant cloned pigs from genome-edited foetal fibroblasts. *Sci Rep* **6**, 24413, doi:10.1038/srep24413 (2016).

92 Ang, Y. S. *et al.* Disease Model of GATA4 Mutation Reveals Transcription Factor Cooperativity in Human Cardiogenesis. *Cell* **167**, 1734-1749.e1722, doi:10.1016/j.cell.2016.11.033 (2016).

93 Wang, G. *et al.* Modeling the mitochondrial cardiomyopathy of Barth syndrome with induced pluripotent stem cell and heart-on-chip technologies. *Nat. Med.* **20**, 616-623, doi:10.1038/nm.3545 (2014).

94 Wallace, E. *et al.* Long QT Syndrome: Genetics and Future Perspective. *Pediatr Cardiol*, doi:10.1007/s00246-019-02151-x (2019).

95 Burnett, J. R. & Hooper, A. J. PCSK9 - A Journey to Cardiovascular Outcomes. *N. Engl. J. Med.* **379**, 2161-2162, doi:10.1056/NEJMe1813758 (2018).

96 Pollin, T. I. *et al.* A null mutation in human APOC3 confers a favorable plasma lipid profile and apparent cardioprotection. *Science* **322**, 1702-1705, doi:10.1126/science.1161524 (2008).

97 Ding, Q. *et al.* Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing. *Circ. Res.* **115**, 488-492, doi:10.1161/circresaha.115.304351 (2014).

98 Crosby, J. *et al.* Loss-of-function mutations in APOC3, triglycerides, and coronary disease. *N. Engl. J. Med.* **371**, 22-31, doi:10.1056/NEJMoa1307095 (2014).

99 Zhan, Y. *et al.* Establishment of a PRKAG2 cardiac syndrome disease model and mechanism study using human induced pluripotent stem cells. *J. Mol. Cell. Cardiol.*, doi:10.1016/j.yjmcc.2018.02.007 (2018).

100 O'Rahilly, S. Human genetics illuminates the paths to metabolic disease. *Nature* **462**, 307-314, doi:10.1038/nature08532 (2009).

101 Coppari, R. & Bjørbæk, C. Leptin revisited: its mechanism of action and potential for treating diabetes. *Nat Rev Drug Discov* **11**, 692-708, doi:10.1038/nrd3757 (2012).

102 Giesbertz, P. *et al.* Metabolite profiling in plasma and tissues of ob/ob and db/db mice identifies novel markers of obesity and type 2 diabetes. *Diabetologia* **58**, 2133-2143, doi:10.1007/s00125-015-3656-y (2015).

103 Chen, Y. *et al.* Generation of obese rat model by transcription activator-like effector nucleases targeting the leptin receptor gene. *Sci China Life Sci*, doi:10.1007/s11427-016-5049-y (2016).

104 Bao, D. *et al.* Preliminary Characterization of a Leptin Receptor Knockout Rat Created by CRISPR/Cas9 System. *Sci Rep* **5**, 15942, doi:10.1038/srep15942 (2015).

105 Wang, X. *et al.* Characterization of novel cytochrome P450 2E1 knockout rat model generated by CRISPR/Cas9. *Biochem. Pharmacol.* **105**, 80-90, doi:10.1016/j.bcp.2016.03.001 (2016).

106 Claussnitzer, M. *et al.* FTO Obesity Variant Circuitry and Adipocyte Browning in Humans. *N. Engl. J. Med.*, doi:10.1056/NEJMoa1502214 (2015).

107 Naylor, J. *et al.* Use of CRISPR/Cas-9 engineered INS-1 pancreatic beta cells to define the pharmacology of dual GIPR/GLP-1R agonists. *Biochem. J.*, doi:10.1042/bcj20160476 (2016).

108 Vethe, H. *et al.* Probing the missing mature β-cell proteomic landscape in differentiating patient iPSC-derived cells. *Sci Rep* **7**, 4780, doi:10.1038/s41598-017-04979-w (2017).

109 Liao, H. K. *et al.* In Vivo Target Gene Activation via CRISPR/Cas9-Mediated Trans-epigenetic Modulation. *Cell*, doi:10.1016/j.cell.2017.10.025 (2017).

110 Tirronen, A., Hokkanen, K., Vuorio, T. & Ylä-Herttuala, S. Recent advances in novel therapies for lipid disorders. *Hum. Mol. Genet.*, doi:10.1093/hmg/ddz132 (2019).

111 Nakagawa, Y. *et al.* Hyperlipidemia and hepatitis in liver-specific CREB3L3 knockout mice generated using a one-step CRISPR/Cas9 system. *Sci Rep* **6**, 27857, doi:10.1038/srep27857 (2016).

112 Carlson, D. F. *et al.* Efficient TALEN-mediated gene knockout in livestock. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 17382-17387, doi:10.1073/pnas.1211446109 (2012).

113 Burkhardt, R. *et al.* Trib1 is a lipid- and myocardial infarction-associated gene that regulates hepatic lipogenesis and VLDL production in mice. *J. Clin. Invest.* **120**, 4410-4414, doi:10.1172/jci44213 (2010).

114 Nagiec, M. M. *et al.* Modulators of hepatic lipoprotein metabolism identified in a search for small-molecule inducers of tribbles pseudokinase 1 expression. *PLoS ONE* **10**, e0120295, doi:10.1371/journal.pone.0120295 (2015).

115 Nance, M. A. Genetics of Huntington disease. *Handb Clin Neurol* **144**, 3-14, doi:10.1016/B978-0-12-801893-4.00001-8 (2017).

116 Wood, L. B., Winslow, A. R. & Strasser, S. D. Systems biology of neurodegenerative diseases. *Integr Biol (Camb)* **7**, 758-775, doi:10.1039/c5ib00031a (2015).

117 Soto, C. & Pritzkow, S. Protein misfolding, aggregation, and conformational strains in neurodegenerative diseases. *Nat. Neurosci.*, doi:10.1038/s41593-018-0235-9 (2018).

118 Hu, Z., Yang, B., Mo, X. & Xiao, H. Mechanism and Regulation of Autophagy and Its Role in Neuronal Diseases. *Mol. Neurobiol.* **52**, 1190-1209, doi:10.1007/s12035-014-8921-4 (2015).

119 Sahebkar, A., Panahi, Y., Yaribeygi, H. & Javadi, B. Oxidative stress in neurodegenerative diseases: a review. *CNS Neurol Disord Drug Targets*, doi:10.2174/1871527317666180425122557 (2018).

120 Rossi, F. & Cattaneo, E. Opinion: neural stem cell therapy for neurological diseases: dreams and reality. *Nat. Rev. Neurosci.* **3**, 401-409, doi:10.1038/nrn809 (2002).

121 Fan, H. C. *et al.* The Role of Gene Editing in Neurodegenerative Diseases. *Cell Transplant* **27**, 364-378, doi:10.1177/0963689717753378 (2018).

122 Garriga-Canut, M. *et al.* Synthetic zinc finger repressors reduce mutant huntingtin expression in the brain of R6/2 mice. *Proc. Natl. Acad. Sci. U.S.A.* **109**, E3136-3145, doi:10.1073/pnas.1206506109 (2012).

123 An, M. C. *et al.* Genetic correction of Huntington's disease phenotypes in induced pluripotent stem cells. *Cell Stem Cell* **11**, 253-263, doi:10.1016/j.stem.2012.04.026 (2012).

124 Xu, X. *et al.* Reversal of Phenotypic Abnormalities by CRISPR/Cas9-Mediated Gene Correction in Huntington Disease Patient-Derived Induced Pluripotent Stem Cells. *Stem Cell Reports*, doi:10.1016/j.stemcr.2017.01.022 (2017).

125 Yan, S. *et al.* A Huntingtin Knockin Pig Model Recapitulates Features of Selective Neurodegeneration in Huntington's Disease. *Cell*, doi:10.1016/j.cell.2018.03.005 (2018).

126 Di Fede, G. *et al.* A recessive mutation in the APP gene with dominant-negative effect on amyloidogenesis. *Science* **323**, 1473-1477, doi:10.1126/science.1168979 (2009).

127 Jonsson, T. *et al.* A mutation in APP protects against Alzheimer's disease and age-related cognitive decline. *Nature* **488**, 96-99, doi:10.1038/nature11283 (2012).

128 Giaccone, G. *et al.* Neuropathology of the recessive A673V APP mutation: Alzheimer disease with distinctive features. *Acta Neuropathol.* **120**, 803-812, doi:10.1007/s00401-010-0747-1 (2010).

129 Martiskainen, H. *et al.* Decreased plasma β-amyloid in the Alzheimer's disease APP A673T variant carriers. *Ann. Neurol.*, doi:10.1002/ana.24969 (2017).

130 Wang, Y. *et al.* Lost region in amyloid precursor protein (APP) through TALEN-mediated genome editing alters mitochondrial morphology. *Sci Rep* **6**, 22244, doi:10.1038/srep22244 (2016).

131 Paquet, D. *et al.* Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. *Nature* **533**, 125-129, doi:10.1038/nature17664 (2016).

132 Abudayyeh, O. O. *et al.* A cytosine deaminase for programmable single-base RNA editing. *Science*, doi:10.1126/science.aax7063 (2019).

133 Lubbe, S. & Morris, H. R. Recent advances in Parkinson’s disease genetics. *J. Neurol.* **261**, 259-266, doi:10.1007/s00415-013-7003-2 (2014).

134 Dansithong, W., Paul, S., Scoles, D. R., Pulst, S. M. & Huynh, D. P. Generation of SNCA Cell Models Using Zinc Finger Nuclease (ZFN) Technology for Efficient High-Throughput Drug Screening. *PLoS ONE* **10**, e0136930, doi:10.1371/journal.pone.0136930 (2015).

135 Reinhardt, P. *et al.* Genetic correction of a LRRK2 mutation in human iPSCs links parkinsonian neurodegeneration to ERK-dependent changes in gene expression. *Cell Stem Cell* **12**, 354-367, doi:10.1016/j.stem.2013.01.008 (2013).

136 Soldner, F. *et al.* Parkinson-associated risk variant in distal enhancer of α-synuclein modulates target gene expression. *Nature* **533**, 95-99, doi:10.1038/nature17939 (2016).

137 Chen, S., Yu, X. & Guo, D. CRISPR-Cas Targeting of Host Genes as an Antiviral Strategy. *Viruses* **10**, doi:10.3390/v10010040 (2018).

138 Perez, E. E. *et al.* Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nat. Biotechnol.* **26**, 808-816, doi:10.1038/nbt1410 (2008).

139 Hütter, G. *et al.* Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N. Engl. J. Med.* **360**, 692-698, doi:10.1056/NEJMoa0802905 (2009).

140 Allers, K. *et al.* Evidence for the cure of HIV infection by CCR5Δ32/Δ32 stem cell transplantation. *Blood* **117**, 2791-2799, doi:10.1182/blood-2010-09-309591 (2011).

141 Allen, A. G. *et al.* Gene Editing of HIV-1 Co-receptors to Prevent and/or Cure Virus Infection. *Front Microbiol* **9**, 2940, doi:10.3389/fmicb.2018.02940 (2018).

142 Didigu, C. A. *et al.* Simultaneous zinc-finger nuclease editing of the HIV coreceptors ccr5 and cxcr4 protects CD4+ T cells from HIV-1 infection. *Blood* **123**, 61-69, doi:10.1182/blood-2013-08-521229 (2014).

143 Mussolino, C. *et al.* TALENs facilitate targeted genome editing in human cells with high specificity and low cytotoxicity. *Nucleic Acids Res.* **42**, 6762-6773, doi:10.1093/nar/gku305 (2014).

144 Ebina, H., Misawa, N., Kanemura, Y. & Koyanagi, Y. Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. *Sci Rep* **3**, 2510, doi:10.1038/srep02510 (2013).

145 Mandal, P. K. *et al.* Efficient ablation of genes in human hematopoietic stem and effector cells using CRISPR/Cas9. *Cell Stem Cell* **15**, 643-652, doi:10.1016/j.stem.2014.10.004 (2014).

146 Hendel, A. *et al.* Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat. Biotechnol.*, doi:10.1038/nbt.3290 (2015).

147 Hu, W. *et al.* RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 11461-11466, doi:10.1073/pnas.1405186111 (2014).

148 Moody, C. A. & Laimins, L. A. Human papillomavirus oncoproteins: pathways to transformation. *Nat. Rev. Cancer* **10**, 550-560, doi:10.1038/nrc2886 (2010).

149 Ding, W. *et al.* Zinc finger nucleases targeting the human papillomavirus E7 oncogene induce E7 disruption and a transformed phenotype in HPV16/18-positive cervical cancer cells. *Clin Cancer Res* **20**, 6495-6503, doi:10.1158/1078-0432.CCR-14-0250 (2014).

150 Ren, C. *et al.* Zinc Finger Nuclease Combines with Cisplatin and Trichostatin A Enhances the Antitumor Potency in Cervical Cancer Cells. *Anticancer Agents Med Chem*, doi:10.2174/1871520618666180509152222 (2018).

151 Wayengera, M. Zinc finger arrays binding human papillomavirus types 16 and 18 genomic DNA: precursors of gene-therapeutics for in-situ reversal of associated cervical neoplasia. *Theor Biol Med Model* **9**, 30, doi:10.1186/1742-4682-9-30 (2012).

152 Hu, Z. *et al.* TALEN-mediated targeting of HPV oncogenes ameliorates HPV-related cervical malignancy. *J Clin Invest* **125**, 425-436, doi:10.1172/JCI78206 (2015).

153 Zhen, S. *et al.* In Vitro and In Vivo Synergistic Therapeutic Effect of Cisplatin with Human Papillomavirus16 E6/E7 CRISPR/Cas9 on Cervical Cancer Cell Line. *Transl Oncol* **9**, 498-504, doi:10.1016/j.tranon.2016.10.002 (2016).

154 Cradick, T. J., Keck, K., Bradshaw, S., Jamieson, A. C. & McCaffrey, A. P. Zinc-finger nucleases as a novel therapeutic strategy for targeting hepatitis B virus DNAs. *Mol. Ther.* **18**, 947-954, doi:10.1038/mt.2010.20 (2010).

155 Ramanan, V. *et al.* CRISPR/Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus. *Sci Rep* **5**, 10833, doi:10.1038/srep10833 (2015).

156 Wang, J. & Quake, S. R. RNA-guided endonuclease provides a therapeutic strategy to cure latent herpesviridae infection. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 13157-13162, doi:10.1073/pnas.1410785111 (2014).

157 Cai, S. W., Zhang, Y., Hou, M. Z., Liu, Y. & Li, X. R. [The research advances and applications of genome editing in hereditary eye diseases]. *Zhonghua Yan Ke Za Zhi* **53**, 386-391, doi:10.3760/cma.j.issn.0412-4081.2017.05.014 (2017).

158 Bjork, S., Hurt, C. M., Ho, V. K. & Angelotti, T. REEPs are membrane shaping adapter proteins that modulate specific g protein-coupled receptor trafficking by affecting ER cargo capacity. *PloS one* **8**, e76366, doi:10.1371/journal.pone.0076366 (2013).

159 Arno, G. *et al.* Mutations in REEP6 Cause Autosomal-Recessive Retinitis Pigmentosa. *American journal of human genetics* **99**, 1305-1315, doi:10.1016/j.ajhg.2016.10.008 (2016).

160 Bowes, C. *et al.* Retinal degeneration in the rd mouse is caused by a defect in the beta subunit of rod cGMP-phosphodiesterase. *Nature* **347**, 677-680, doi:10.1038/347677a0 (1990).

161 Keeler, C. E. THE GEOTROPIC REACTION OF RODLESS MICE IN LIGHT AND IN DARKNESS. *The Journal of general physiology* **11**, 361-368, doi:10.1085/jgp.11.4.361 (1928).

162 Wu, W. H. *et al.* CRISPR Repair Reveals Causative Mutation in a Preclinical Model of Retinitis Pigmentosa. *Molecular therapy : the journal of the American Society of Gene Therapy* **24**, 1388-1394, doi:10.1038/mt.2016.107 (2016).

163 McGill, T. J. *et al.* Optomotor and immunohistochemical changes in the juvenile S334ter rat. *Experimental eye research* **104**, 65-73, doi:10.1016/j.exer.2012.09.006 (2012).

164 Cho, G. Y. *et al.* CRISPR-mediated Ophthalmic Genome Surgery. *Curr Ophthalmol Rep* **5**, 199-206, doi:10.1007/s40135-017-0144-1 (2017).

165 Bakondi, B. *et al.* In Vivo CRISPR/Cas9 Gene Editing Corrects Retinal Dystrophy in the S334ter-3 Rat Model of Autosomal Dominant Retinitis Pigmentosa. *Molecular therapy : the journal of the American Society of Gene Therapy* **24**, 556-563, doi:10.1038/mt.2015.220 (2016).

166 Latella, M. C. *et al.* In vivo Editing of the Human Mutant Rhodopsin Gene by Electroporation of Plasmid-based CRISPR/Cas9 in the Mouse Retina. *Molecular therapy. Nucleic acids* **5**, e389, doi:10.1038/mtna.2016.92 (2016).

167 Suzuki, K. *et al.* In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature* **540**, 144-149, doi:10.1038/nature20565 (2016).

168 Bassuk, A. G., Zheng, A., Li, Y., Tsang, S. H. & Mahajan, V. B. Precision Medicine: Genetic Repair of Retinitis Pigmentosa in Patient-Derived Stem Cells. *Sci Rep* **6**, 19969, doi:10.1038/srep19969 (2016).

169 Deng, W. L. *et al.* Gene Correction Reverses Ciliopathy and Photoreceptor Loss in iPSC-Derived Retinal Organoids from Retinitis Pigmentosa Patients. *Stem Cell Reports* **10**, 1267-1281, doi:10.1016/j.stemcr.2018.02.003 (2018).

170 Liao, C., Zhang, D., Mungo, C., Tompkins, D. A. & Zeidan, A. M. Is diabetes mellitus associated with increased incidence and disease-specific mortality in endometrial cancer? A systematic review and meta-analysis of cohort studies. *Gynecologic oncology* **135**, 163-171, doi:10.1016/j.ygyno.2014.07.095 (2014).

171 Zhong, H., Chen, Y., Li, Y., Chen, R. & Mardon, G. CRISPR-engineered mosaicism rapidly reveals that loss of Kcnj13 function in mice mimics human disease phenotypes. *Sci Rep* **5**, 8366, doi:10.1038/srep08366 (2015).

172 Ruan, G. X. *et al.* CRISPR/Cas9-Mediated Genome Editing as a Therapeutic Approach for Leber Congenital Amaurosis 10. *Molecular therapy : the journal of the American Society of Gene Therapy* **25**, 331-341, doi:10.1016/j.ymthe.2016.12.006 (2017).

173 Maeder, M. L. *et al.* Development of a gene-editing approach to restore vision loss in Leber congenital amaurosis type 10. *Nat Med* **25**, 229-233, doi:10.1038/s41591-018-0327-9 (2019).

174 Lee, W. H., Murphree, A. L. & Benedict, W. F. Expression and amplification of the N-myc gene in primary retinoblastoma. *Nature* **309**, 458-460, doi:10.1038/309458a0 (1984).

175 Solin, S. L., Shive, H. R., Woolard, K. D., Essner, J. J. & McGrail, M. Rapid tumor induction in zebrafish by TALEN-mediated somatic inactivation of the retinoblastoma1 tumor suppressor rb1. *Sci Rep* **5**, 13745, doi:10.1038/srep13745 (2015).

176 Naert, T. *et al.* CRISPR/Cas9 mediated knockout of rb1 and rbl1 leads to rapid and penetrant retinoblastoma development in Xenopus tropicalis. *Sci Rep* **6**, 35264, doi:10.1038/srep35264 (2016).

177 Tu, J. *et al.* Generation of human embryonic stem cell line with heterozygous RB1 deletion by CRIPSR/Cas9 nickase. *Stem Cell Res* **28**, 29-32, doi:10.1016/j.scr.2018.01.021 (2018).

178 Hollands, H. *et al.* Do findings on routine examination identify patients at risk for primary open-angle glaucoma? The rational clinical examination systematic review. *Jama* **309**, 2035-2042, doi:10.1001/jama.2013.5099 (2013).

179 Alward, W. L. *et al.* Clinical features associated with mutations in the chromosome 1 open-angle glaucoma gene (GLC1A). *The New England journal of medicine* **338**, 1022-1027, doi:10.1056/nejm199804093381503 (1998).

180 Stone, E. M. *et al.* Identification of a gene that causes primary open angle glaucoma. *Science* **275**, 668-670, doi:10.1126/science.275.5300.668 (1997).

181 Kim, B. S. *et al.* Targeted Disruption of the Myocilin Gene (Myoc) Suggests that Human Glaucoma-Causing Mutations Are Gain of Function. *Molecular and cellular biology* **21**, 7707-7713, doi:10.1128/mcb.21.22.7707-7713.2001 (2001).

182 Carbone, M. A. *et al.* Overexpression of myocilin in the Drosophila eye activates the unfolded protein response: implications for glaucoma. *PloS one* **4**, e4216, doi:10.1371/journal.pone.0004216 (2009).

183 Joe, M. K. *et al.* Accumulation of mutant myocilins in ER leads to ER stress and potential cytotoxicity in human trabecular meshwork cells. *Biochemical and biophysical research communications* **312**, 592-600, doi:10.1016/j.bbrc.2003.10.162 (2003).

184 Liu, Y. & Vollrath, D. Reversal of mutant myocilin non-secretion and cell killing: implications for glaucoma. *Human molecular genetics* **13**, 1193-1204, doi:10.1093/hmg/ddh128 (2004).

185 Yam, G. H., Gaplovska-Kysela, K., Zuber, C. & Roth, J. Aggregated myocilin induces russell bodies and causes apoptosis: implications for the pathogenesis of myocilin-caused primary open-angle glaucoma. *The American journal of pathology* **170**, 100-109, doi:10.2353/ajpath.2007.060806 (2007).

186 Jain, A. *et al.* CRISPR-Cas9-based treatment of myocilin-associated glaucoma. *Proc Natl Acad Sci U S A* **114**, 11199-11204, doi:10.1073/pnas.1706193114 (2017).

187 Liao, H. *et al.* Development of allele-specific therapeutic siRNA in Meesmann epithelial corneal dystrophy. *PloS one* **6**, e28582, doi:10.1371/journal.pone.0028582 (2011).

188 Courtney, D. G. *et al.* CRISPR/Cas9 DNA cleavage at SNP-derived PAM enables both in vitro and in vivo KRT12 mutation-specific targeting. *Gene Ther* **23**, 108-112, doi:10.1038/gt.2015.82 (2016).

189 Shima, M. *et al.* Factor VIII-Mimetic Function of Humanized Bispecific Antibody in Hemophilia A. *N. Engl. J. Med.* **374**, 2044-2053, doi:10.1056/NEJMoa1511769 (2016).

190 Park, C. Y. *et al.* Functional Correction of Large Factor VIII Gene Chromosomal Inversions in Hemophilia A Patient-Derived iPSCs Using CRISPR-Cas9. *Cell Stem Cell* **17**, 213-220, doi:10.1016/j.stem.2015.07.001 (2015).

191 Guan, Y. *et al.* CRISPR/Cas9-mediated somatic correction of a novel coagulator factor IX gene mutation ameliorates hemophilia in mouse. *EMBO Mol Med*, doi:10.15252/emmm.201506039 (2016).

192 Hai, T., Teng, F., Guo, R., Li, W. & Zhou, Q. One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. *Cell Res.* **24**, 372-375, doi:10.1038/cr.2014.11 (2014).

193 Rees, D. C., Williams, T. N. & Gladwin, M. T. Sickle-cell disease. *Lancet* **376**, 2018-2031, doi:10.1016/s0140-6736(10)61029-x (2010).

194 Dever, D. P. *et al.* CRISPR/Cas9 β-globin gene targeting in human haematopoietic stem cells. *Nature*, doi:10.1038/nature20134 (2016).

195 Martin, R. M. *et al.* Highly Efficient and Marker-free Genome Editing of Human Pluripotent Stem Cells by CRISPR-Cas9 RNP and AAV6 Donor-Mediated Homologous Recombination. *Cell Stem Cell* **24**, 821-828.e825, doi:10.1016/j.stem.2019.04.001 (2019).

196 Verhaart, I. E. C. & Aartsma-Rus, A. Therapeutic developments for Duchenne muscular dystrophy. *Nat Rev Neurol*, doi:10.1038/s41582-019-0203-3 (2019).

197 Larcher, T. *et al.* Characterization of dystrophin deficient rats: a new model for Duchenne muscular dystrophy. *PLoS ONE* **9**, e110371, doi:10.1371/journal.pone.0110371 (2014).

198 Sui, T. *et al.* A novel rabbit model of Duchenne muscular dystrophy generated by CRISPR/Cas9. *Dis Model Mech* **11**, doi:10.1242/dmm.032201 (2018).

199 Chen, Y. *et al.* Generation of Cynomolgus Monkey Chimeric Fetuses using Embryonic Stem Cells. *Cell Stem Cell* **17**, 116-124, doi:10.1016/j.stem.2015.06.004 (2015).

200 Chen, Y. *et al.* Functional disruption of the dystrophin gene in rhesus monkey using CRISPR/Cas9. *Hum. Mol. Genet.* **24**, 3764-3774, doi:10.1093/hmg/ddv120 (2015).

201 Xu, L. *et al.* CRISPR-mediated genome editing restores dystrophin expression and function in mdx mice. *Mol. Ther.*, doi:10.1038/mt.2015.192 (2015).

202 Bengtsson, N. E. *et al.* Muscle-specific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy. *Nat Commun* **8**, 14454, doi:10.1038/ncomms14454 (2017).

203 Nelson, C. E. *et al.* In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science*, doi:10.1126/science.aad5143 (2015).

204 Pai, S. Y. *et al.* Transplantation outcomes for severe combined immunodeficiency, 2000-2009. *N. Engl. J. Med.* **371**, 434-446, doi:10.1056/NEJMoa1401177 (2014).

205 Lombardo, A. *et al.* Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat. Biotechnol.* **25**, 1298-1306, doi:10.1038/nbt1353 (2007).

206 Flisikowska, T. *et al.* Efficient immunoglobulin gene disruption and targeted replacement in rabbit using zinc finger nucleases. *PLoS ONE* **6**, e21045, doi:10.1371/journal.pone.0021045 (2011).

207 Wang, Y. *et al.* Generation of knockout rabbits using transcription activator-like effector nucleases. *Cell Regen (Lond)* **3**, 3, doi:10.1186/2045-9769-3-3 (2014).

208 Yan, Q. *et al.* Generation of multi-gene knockout rabbits using the Cas9/gRNA system. *Cell Regen (Lond)* **3**, 12, doi:10.1186/2045-9769-3-12 (2014).

209 Zhou, J. *et al.* One-step generation of different immunodeficient mice with multiple gene modifications by CRISPR/Cas9 mediated genome engineering. *Int. J. Biochem. Cell Biol.* **46**, 49-55, doi:10.1016/j.biocel.2013.10.010 (2014).

210 Ott de Bruin, L. M., Volpi, S. & Musunuru, K. Novel Genome-Editing Tools to Model and Correct Primary Immunodeficiencies. *Front Immunol* **6**, 250, doi:10.3389/fimmu.2015.00250 (2015).

211 Chen, Y. *et al.* Modeling Rett Syndrome Using TALEN-Edited MECP2 Mutant Cynomolgus Monkeys. *Cell* **169**, 945-955.e910, doi:10.1016/j.cell.2017.04.035 (2017).

212 Chen, J. R. *et al.* Effects of genetic correction on the differentiation of hair cell-like cells from iPSCs with MYO15A mutation. *Cell Death Differ.*, doi:10.1038/cdd.2016.16 (2016).

213 Jiang, W. *et al.* Production of Wilson Disease Model Rabbits with Homology-Directed Precision Point Mutations in the ATP7B Gene Using the CRISPR/Cas9 System. *Sci Rep* **8**, 1332, doi:10.1038/s41598-018-19774-4 (2018).

214 Kurome, M. *et al.* 361 growth hormone receptor mutant pigs produced by using the clustered regularly interspaced short palindromic repeats (crispr) and crispr-associated systems in in vitro-produced zygotes. *Reprod. Fertil. Dev.* **27**, 269, doi:10.1071/RDv27n1Ab361 (2014).

215 Tseng, W. C. *et al.* Modeling Niemann-Pick disease type C1 in zebrafish: a robust platform for in vivo screening of candidate therapeutic compounds. *Dis Model Mech* **11**, doi:10.1242/dmm.034165 (2018).

216 Abdul-Wahab, A., Qasim, W. & McGrath, J. A. Gene therapies for inherited skin disorders. *Semin Cutan Med Surg* **33**, 83-90 (2014).

217 Kirkwood, J. M. *et al.* Immunotherapy of cancer in 2012. *CA Cancer J Clin* **62**, 309-335, doi:10.3322/caac.20132 (2012).

218 Rein, L. A. M., Yang, H. & Chao, N. J. Applications of Gene Editing Technologies to Cellular Therapies. *Biol Blood Marrow Transplant* **24**, 1537-1545, doi:10.1016/j.bbmt.2018.03.021 (2018).

219 June, C. H. & Sadelain, M. Chimeric Antigen Receptor Therapy. *N Engl J Med* **379**, 64-73, doi:10.1056/NEJMra1706169 (2018).

220 Sadelain, M., Brentjens, R. & Rivière, I. The promise and potential pitfalls of chimeric antigen receptors. *Curr. Opin. Immunol.* **21**, 215-223, doi:10.1016/j.coi.2009.02.009 (2009).

221 Davenport, A. J. *et al.* CAR-T Cells Inflict Sequential Killing of Multiple Tumor Target Cells. *Cancer Immunol Res* **3**, 483-494, doi:10.1158/2326-6066.Cir-15-0048 (2015).

222 Zhao, J., Lin, Q., Song, Y. & Liu, D. Universal CARs, universal T cells, and universal CAR T cells. *J Hematol Oncol* **11**, 132, doi:10.1186/s13045-018-0677-2 (2018).

223 Torikai, H. *et al.* A foundation for universal T-cell based immunotherapy: T cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. *Blood* **119**, 5697-5705, doi:10.1182/blood-2012-01-405365 (2012).

224 Torikai, H. *et al.* Toward eliminating HLA class I expression to generate universal cells from allogeneic donors. *Blood* **122**, 1341-1349, doi:10.1182/blood-2013-03-478255 (2013).

225 Qasim, W. *et al.* Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. *Sci Transl Med* **9**, doi:10.1126/scitranslmed.aaj2013 (2017).

226 Wei, G., Wang, J., Huang, H. & Zhao, Y. Novel immunotherapies for adult patients with B-lineage acute lymphoblastic leukemia. *J Hematol Oncol* **10**, 150, doi:10.1186/s13045-017-0516-x (2017).

227 Liu, X. *et al.* CRISPR-Cas9-mediated multiplex gene editing in CAR-T cells. *Cell Res.*, doi:10.1038/cr.2016.142 (2016).

228 Ren, J. *et al.* A versatile system for rapid multiplex genome-edited CAR T cell generation. *Oncotarget*, doi:10.18632/oncotarget.15218 (2017).

229 Kenderian, S. S. *et al.* Identification of PD1 and TIM3 As Checkpoints That Limit Chimeric Antigen Receptor T Cell Efficacy in Leukemia. *Biology of Blood and Marrow Transplantation* **22**, S19-S21, doi:10.1016/j.bbmt.2015.11.291 (2016).

230 Leone, R. D. & Emens, L. A. Targeting adenosine for cancer immunotherapy. *J Immunother Cancer* **6**, 57, doi:10.1186/s40425-018-0360-8 (2018).

231 Zhang, Y. *et al.* CRISPR-Cas9 mediated LAG-3 disruption in CAR-T cells. *Front Med*, doi:10.1007/s11684-017-0543-6 (2017).

232 Wong, A. S. *et al.* Multiplexed barcoded CRISPR-Cas9 screening enabled by CombiGEM. *Proc. Natl. Acad. Sci. U.S.A.*, doi:10.1073/pnas.1517883113 (2016).

233 Yu, J. S. L. & Yusa, K. Genome-wide CRISPR-Cas9 Screening in Mammalian Cells. *Methods*, doi:10.1016/j.ymeth.2019.04.015 (2019).

234 Luo, J. CRISPR/Cas9: From Genome Engineering to Cancer Drug Discovery. *Trends Cancer* **2**, 313-324, doi:10.1016/j.trecan.2016.05.001 (2016).

235 Bester, A. C. *et al.* An Integrated Genome-wide CRISPRa Approach to Functionalize lncRNAs in Drug Resistance. *Cell* **173**, 649-664.e620, doi:10.1016/j.cell.2018.03.052 (2018).

236 Tzelepis, K. *et al.* A CRISPR Dropout Screen Identifies Genetic Vulnerabilities and Therapeutic Targets in Acute Myeloid Leukemia. *Cell Rep* **17**, 1193-1205, doi:10.1016/j.celrep.2016.09.079 (2016).

237 Munoz, D. M. *et al.* CRISPR screens provide a comprehensive assessment of cancer vulnerabilities but generate false-positive hits for highly amplified genomic regions. *Cancer Discov*, doi:10.1158/2159-8290.Cd-16-0178 (2016).

238 Hart, T. *et al.* High-Resolution CRISPR Screens Reveal Fitness Genes and Genotype-Specific Cancer Liabilities. *Cell* **163**, 1515-1526, doi:10.1016/j.cell.2015.11.015 (2015).

239 Wang, T. *et al.* Gene Essentiality Profiling Reveals Gene Networks and Synthetic Lethal Interactions with Oncogenic Ras. *Cell*, doi:10.1016/j.cell.2017.01.013 (2017).

240 Chen, S. *et al.* Genome-wide CRISPR screen in a mouse model of tumor growth and metastasis. *Cell* **160**, 1246-1260, doi:10.1016/j.cell.2015.02.038 (2015).

241 McFadden, D. G. *et al.* Genetic and clonal dissection of murine small cell lung carcinoma progression by genome sequencing. *Cell* **156**, 1298-1311, doi:10.1016/j.cell.2014.02.031 (2014).

242 Tang, J. T. *et al.* MicroRNA 345, a methylation-sensitive microRNA is involved in cell proliferation and invasion in human colorectal cancer. *Carcinogenesis* **32**, 1207-1215, doi:10.1093/carcin/bgr114 (2011).

243 Cheng, Z., Ma, R., Tan, W. & Zhang, L. MiR-152 suppresses the proliferation and invasion of NSCLC cells by inhibiting FGF2. *Exp. Mol. Med.* **46**, e112, doi:10.1038/emm.2014.51 (2014).

244 Song, C. Q. *et al.* Genome-wide CRISPR Screen Identifies Regulators of MAPK as Suppressors of Liver Tumors in Mice. *Gastroenterology*, doi:10.1053/j.gastro.2016.12.002 (2016).

245 Han, K. *et al.* Synergistic drug combinations for cancer identified in a CRISPR screen for pairwise genetic interactions. *Nat. Biotechnol.*, doi:10.1038/nbt.3834 (2017).

246 Liu, S. J. *et al.* CRISPRi-based genome-scale identification of functional long noncoding RNA loci in human cells. *Science*, doi:10.1126/science.aah7111 (2016).

247 Zhu, S. *et al.* Genome-scale deletion screening of human long non-coding RNAs using a paired-guide RNA CRISPR-Cas9 library. *Nat. Biotechnol.*, doi:10.1038/nbt.3715 (2016).

248 Esposito, R. *et al.* Hacking the Cancer Genome: Profiling Therapeutically Actionable Long Non-coding RNAs Using CRISPR-Cas9 Screening. *Cancer Cell* **35**, 545-557, doi:10.1016/j.ccell.2019.01.019 (2019).

249 Boettcher, M. *et al.* Dual gene activation and knockout screen reveals directional dependencies in genetic networks. *Nat. Biotechnol.*, doi:10.1038/nbt.4062 (2018).

250 Yuan, Z. *et al.* Olaparib hydroxamic acid derivatives as dual PARP and HDAC inhibitors for cancer therapy. *Bioorg. Med. Chem.*, doi:10.1016/j.bmc.2017.05.058 (2017).

251 Shen, J. P. *et al.* Combinatorial CRISPR-Cas9 screens for de novo mapping of genetic interactions. *Nat. Methods*, doi:10.1038/nmeth.4225 (2017).

252 Baliou, S. *et al.* CRISPR therapeutic tools for complex genetic disorders and cancer (Review). *Int. J. Oncol.*, doi:10.3892/ijo.2018.4434 (2018).

253 Shalem, O., Sanjana, N. E. & Zhang, F. High-throughput functional genomics using CRISPR-Cas9. *Nat. Rev. Genet.* **16**, 299-311, doi:10.1038/nrg3899 (2015).

254 Heigwer, F. *et al.* CRISPR library designer (CLD): software for multispecies design of single guide RNA libraries. *Genome Biol.* **17**, 55, doi:10.1186/s13059-016-0915-2 (2016).

255 Ruiz, S. *et al.* A Genome-wide CRISPR Screen Identifies CDC25A as a Determinant of Sensitivity to ATR Inhibitors. *Mol Cell* **62**, 307-313, doi:10.1016/j.molcel.2016.03.006 (2016).

256 Krall, E. B. *et al.* KEAP1 loss modulates sensitivity to kinase targeted therapy in lung cancer. *Elife* **6**, doi:10.7554/eLife.18970 (2017).

257 Manguso, R. T. *et al.* In vivo CRISPR screening identifies Ptpn2 as a cancer immunotherapy target. *Nature*, doi:10.1038/nature23270 (2017).

258 Flynn, R. *et al.* CRISPR-mediated genotypic and phenotypic correction of a chronic granulomatous disease mutation in human iPS cells. *Exp. Hematol.* **43**, 838-848.e833, doi:10.1016/j.exphem.2015.06.002 (2015).

259 Rahman, N. Mainstreaming genetic testing of cancer predisposition genes. *Clin Med* **14**, 436-439, doi:10.7861/clinmedicine.14-4-436 (2014).

260 Gootenberg, J. S. *et al.* Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science*, doi:10.1126/science.aam9321 (2017).

261 Chen, J. S. *et al.* CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science*, doi:10.1126/science.aar6245 (2018).

262 Chertow, D. S. Next-generation diagnostics with CRISPR. *Science* **360**, 381-382, doi:10.1126/science.aat4982 (2018).

263 Yang, H. *et al.* Break Breast Cancer Addiction by CRISPR/Cas9 Genome Editing. *J Cancer* **9**, 219-231, doi:10.7150/jca.22554 (2018).

264 Wang, Y. *et al.* The BRCA1-Δ11q Alternative Splice Isoform Bypasses Germline Mutations and Promotes Therapeutic Resistance to PARP Inhibition and Cisplatin. *Cancer Res.* **76**, 2778-2790, doi:10.1158/0008-5472.Can-16-0186 (2016).

265 Cyranoski, D. CRISPR gene-editing tested in a person for the first time. *Nature* **539**, 479, doi:10.1038/nature.2016.20988 (2016).

266 Lázaro, C., Ravella, A., Gaona, A., Volpini, V. & Estivill, X. Neurofibromatosis type 1 due to germ-line mosaicism in a clinically normal father. *N. Engl. J. Med.* **331**, 1403-1407, doi:10.1056/nejm199411243312102 (1994).

267 Wilen, C. B. *et al.* Engineering HIV-resistant human CD4+ T cells with CXCR4-specific zinc-finger nucleases. *PLoS Pathog.* **7**, e1002020, doi:10.1371/journal.ppat.1002020 (2011).

268 Lunzen, J. V. *et al.* Transfer of Autologous Gene-modified T Cells in HIV-infected Patients with Advanced Immunodeficiency and Drug-resistant Virus. *Mol. Ther.* **15**, 1024-1033, doi:10.1038/mt.sj.6300124 (2007).

269 Voit, R. A., McMahon, M. A., Sawyer, S. L. & Porteus, M. H. Generation of an HIV resistant T-cell line by targeted "stacking" of restriction factors. *Mol. Ther.* **21**, 786-795, doi:10.1038/mt.2012.284 (2013).

270 Tebas, P. *et al.* Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N. Engl. J. Med.* **370**, 901-910, doi:10.1056/NEJMoa1300662 (2014).

271 Xu, L. *et al.* CRISPR-Edited Stem Cells in a Patient with HIV and Acute Lymphocytic Leukemia. *N. Engl. J. Med.*, doi:10.1056/NEJMoa1817426 (2019).

272 Choi, K. D. *et al.* Hematopoietic and endothelial differentiation of human induced pluripotent stem cells. *Stem Cells* **27**, 559-567, doi:10.1634/stemcells.2008-0922 (2009).

273 Dolan, G. *et al.* Haemophilia B: Where are we now and what does the future hold? *Blood Rev* **32**, 52-60, doi:10.1016/j.blre.2017.08.007 (2018).

274 Origa, R. beta-Thalassemia. *Genet Med* **19**, 609-619, doi:10.1038/gim.2016.173 (2017).

275 Bauer, D. E. & Orkin, S. H. Hemoglobin switching's surprise: the versatile transcription factor BCL11A is a master repressor of fetal hemoglobin. *Curr Opin Genet Dev* **33**, 62-70, doi:10.1016/j.gde.2015.08.001 (2015).

276 Moore, C. B. T., Christie, K. A., Marshall, J. & Nesbit, M. A. Personalised genome editing - The future for corneal dystrophies. *Prog Retin Eye Res* **65**, 147-165, doi:10.1016/j.preteyeres.2018.01.004 (2018).

277 Wen, W. S., Yuan, Z. M., Ma, S. J., Xu, J. & Yuan, D. T. CRISPR-Cas9 systems: versatile cancer modelling platforms and promising therapeutic strategies. *Int. J. Cancer* **138**, 1328-1336, doi:10.1002/ijc.29626 (2016).

278 Fu, Y. *et al.* High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* **31**, 822-826, doi:10.1038/nbt.2623 (2013).

279 Guilinger, J. P. *et al.* Broad specificity profiling of TALENs results in engineered nucleases with improved DNA-cleavage specificity. *Nat. Methods* **11**, 429-435, doi:10.1038/nmeth.2845 (2014).

280 Pattanayak, V., Ramirez, C. L., Joung, J. K. & Liu, D. R. Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. *Nat. Methods* **8**, 765-770, doi:10.1038/nmeth.1670 (2011).

281 Vouillot, L., Thélie, A. & Pollet, N. Comparison of T7E1 and Surveyor Mismatch Cleavage Assays to Detect Mutations Triggered by Engineered Nucleases. *G3 (Bethesda)* **5**, 407-415, doi:10.1534/g3.114.015834 (2015).

282 Pattanayak, V. *et al.* High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat Biotechnol* **31**, 839-843, doi:10.1038/nbt.2673 (2013).

283 Seeger, C. & Sohn, J. A. Complete spectrum of CRISPR/Cas9-induced mutations on HBV cccDNA. *Mol. Ther.*, doi:10.1038/mt.2016.94 (2016).

284 Gabriel, R. *et al.* An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nat. Biotechnol.* **29**, 816-823, doi:10.1038/nbt.1948 (2011).

285 Osborn, M. J. *et al.* Evaluation of TCR Gene Editing achieved by TALENs, CRISPR/Cas9 and megaTAL nucleases. *Mol. Ther.*, doi:10.1038/mt.2015.197 (2015).

286 Hsu, P. D. *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* **31**, 827-832, doi:10.1038/nbt.2647 (2013).

287 Mali, P. *et al.* CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat. Biotechnol.* **31**, 833-838, doi:10.1038/nbt.2675 (2013).

288 Zetsche, B., Volz, S. E. & Zhang, F. A split-Cas9 architecture for inducible genome editing and transcription modulation. *Nat. Biotechnol.* **33**, 139-142, doi:10.1038/nbt.3149 (2015).

289 Moon, S. B., Kim, D. Y., Ko, J. H., Kim, J. S. & Kim, Y. S. Improving CRISPR Genome Editing by Engineering Guide RNAs. *Trends Biotechnol.*, doi:10.1016/j.tibtech.2019.01.009 (2019).

290 Cho, S. W. *et al.* Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res* **24**, 132-141, doi:10.1101/gr.162339.113 (2014).

291 Xie, S., Shen, B., Zhang, C., Huang, X. & Zhang, Y. sgRNAcas9: a software package for designing CRISPR sgRNA and evaluating potential off-target cleavage sites. *PLoS ONE* **9**, e100448, doi:10.1371/journal.pone.0100448 (2014).

292 Sander, J. D. *et al.* ZiFiT (Zinc Finger Targeter): an updated zinc finger engineering tool. *Nucleic Acids Res* **38**, W462-468, doi:10.1093/nar/gkq319 (2010).

293 Kiani, S. *et al.* CRISPR transcriptional repression devices and layered circuits in mammalian cells. *Nat. Methods* **11**, 723-726, doi:10.1038/nmeth.2969 (2014).

294 Slaymaker, I. M. *et al.* Rationally engineered Cas9 nucleases with improved specificity. *Science*, doi:10.1126/science.aad5227 (2015).

295 Kleinstiver, B. P. *et al.* High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature*, doi:10.1038/nature16526 (2016).

296 Kim, S., Kim, D., Cho, S. W., Kim, J. & Kim, J. S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res.* **24**, 1012-1019, doi:10.1101/gr.171322.113 (2014).

297 Suresh, B., Ramakrishna, S. & Kim, H. Cell-Penetrating Peptide-Mediated Delivery of Cas9 Protein and Guide RNA for Genome Editing. *Methods Mol. Biol.* **1507**, 81-94, doi:10.1007/978-1-4939-6518-2\_7 (2017).

298 Dong, L. *et al.* An anti-CRISPR protein disables type V Cas12a by acetylation. *Nat Struct Mol Biol* **26**, 308-314, doi:10.1038/s41594-019-0206-1 (2019).

299 Shin, J. *et al.* Disabling Cas9 by an anti-CRISPR DNA mimic. *Sci Adv* **3**, e1701620, doi:10.1126/sciadv.1701620 (2017).

300 Shrivastav, M., De Haro, L. P. & Nickoloff, J. A. Regulation of DNA double-strand break repair pathway choice. *Cell Res.* **18**, 134-147, doi:10.1038/cr.2007.111 (2008).

301 Ciccia, A. & Elledge, S. J. The DNA damage response: making it safe to play with knives. *Mol. Cell* **40**, 179-204, doi:10.1016/j.molcel.2010.09.019 (2010).

302 Chapman, J. R., Taylor, M. R. & Boulton, S. J. Playing the end game: DNA double-strand break repair pathway choice. *Mol. Cell* **47**, 497-510, doi:10.1016/j.molcel.2012.07.029 (2012).

303 Maruyama, T. *et al.* Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat Biotechnol* **33**, 538-542, doi:10.1038/nbt.3190 (2015).

304 Chu, V. T. *et al.* Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nat. Biotechnol.*, doi:10.1038/nbt.3198 (2015).

305 Canny, M. D. *et al.* Inhibition of 53BP1 favors homology-dependent DNA repair and increases CRISPR-Cas9 genome-editing efficiency. *Nat. Biotechnol.*, doi:10.1038/nbt.4021 (2017).

306 Song, J. *et al.* RS-1 enhances CRISPR/Cas9- and TALEN-mediated knock-in efficiency. *Nat Commun* **7**, 10548, doi:10.1038/ncomms10548 (2016).

307 Gao, Y. *et al.* Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability and development. *Nature* **404**, 897-900, doi:10.1038/35009138 (2000).

308 Moshous, D. *et al.* Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency. *Cell* **105**, 177-186 (2001).

309 Shah, S. Z. *et al.* Advances In Research On Genome Editing Crispr-Cas9 Technology. *J Ayub Med Coll Abbottabad* **31**, 108-122 (2019).

310 Lin, S., Staahl, B. T., Alla, R. K. & Doudna, J. A. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *Elife* **3**, e04766, doi:10.7554/eLife.04766 (2014).

311 Kim, K. *et al.* Highly efficient RNA-guided base editing in mouse embryos. *Nat. Biotechnol.*, doi:10.1038/nbt.3816 (2017).

312 Zafra, M. P. *et al.* Optimized base editors enable efficient editing in cells, organoids and mice. *Nat. Biotechnol.*, doi:10.1038/nbt.4194 (2018).

313 Grünewald, J. *et al.* CRISPR DNA base editors with reduced RNA off-target and self-editing activities. *Nat. Biotechnol.*, doi:10.1038/s41587-019-0236-6 (2019).

314 Zong, Y. *et al.* Efficient C-to-T base editing in plants using a fusion of nCas9 and human APOBEC3A. *Nat. Biotechnol.*, doi:10.1038/nbt.4261 (2018).

315 Hur, J. K. *et al.* Targeted mutagenesis in mice by electroporation of Cpf1 ribonucleoproteins. *Nat. Biotechnol.*, doi:10.1038/nbt.3596 (2016).

316 Gori, J. L. *et al.* Delivery and Specificity of CRISPR-Cas9 Genome Editing Technologies for Human Gene Therapy. *Hum. Gene Ther.*, doi:10.1089/hum.2015.074 (2015).

317 Zuris, J. A. *et al.* Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. *Nat. Biotechnol.* **33**, 73-80, doi:10.1038/nbt.3081 (2015).

318 Mout, R., Ray, M., Lee, Y. W., Scaletti, F. & Rotello, V. M. In Vivo Delivery of CRISPR/Cas9 for Therapeutic Gene Editing: Progress and Challenges. *Bioconjug. Chem.*, doi:10.1021/acs.bioconjchem.7b00057 (2017).

319 Yin, H., Kauffman, K. J. & Anderson, D. G. Delivery technologies for genome editing. *Nat Rev Drug Discov* **16**, 387-399, doi:10.1038/nrd.2016.280 (2017).

320 Kotterman, M. A. & Schaffer, D. V. Engineering adeno-associated viruses for clinical gene therapy. *Nat. Rev. Genet.* **15**, 445-451, doi:10.1038/nrg3742 (2014).

321 Maggio, I. *et al.* Adenoviral vector delivery of RNA-guided CRISPR/Cas9 nuclease complexes induces targeted mutagenesis in a diverse array of human cells. *Sci Rep* **4**, 5105, doi:10.1038/srep05105 (2014).

322 Feng, M. *et al.* Stable in vivo gene transduction via a novel adenoviral/retroviral chimeric vector. *Nat. Biotechnol.* **15**, 866-870, doi:10.1038/nbt0997-866 (1997).

323 Koike-Yusa, H., Li, Y., Tan, E. P., Velasco-Herrera Mdel, C. & Yusa, K. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat. Biotechnol.* **32**, 267-273, doi:10.1038/nbt.2800 (2014).

324 Paulk, N. K. *et al.* Adeno-associated virus gene repair corrects a mouse model of hereditary tyrosinemia in vivo. *Hepatology* **51**, 1200-1208, doi:10.1002/hep.23481 (2010).

325 Charlesworth, C. T. *et al.* Identification of pre-existing adaptive immunity to Cas9 proteins in humans. *bioRxiv*, doi:10.1101/243345 (2018).

326 Ihry, R. J. *et al.* p53 inhibits CRISPR-Cas9 engineering in human pluripotent stem cells. *Nat. Med.*, doi:10.1038/s41591-018-0050-6 (2018).

327 Liang, C. *et al.* Tumor cell-targeted delivery of CRISPR/Cas9 by aptamer-functionalized lipopolymer for therapeutic genome editing of VEGFA in osteosarcoma. *Biomaterials* **147**, 68-85, doi:10.1016/j.biomaterials.2017.09.015 (2017).

328 Luo, Y. L. *et al.* Macrophage-Specific in Vivo Gene Editing Using Cationic Lipid-Assisted Polymeric Nanoparticles. *ACS Nano*, doi:10.1021/acsnano.7b07874 (2018).

329 Finn, J. D. *et al.* A Single Administration of CRISPR/Cas9 Lipid Nanoparticles Achieves Robust and Persistent In Vivo Genome Editing. *Cell Rep* **22**, 2227-2235, doi:10.1016/j.celrep.2018.02.014 (2018).

330 Wang, H. X. *et al.* Nonviral gene editing via CRISPR/Cas9 delivery by membrane-disruptive and endosomolytic helical polypeptide. *Proc Natl Acad Sci U S A* **115**, 4903-4908, doi:10.1073/pnas.1712963115 (2018).

331 Ramakrishna, S. *et al.* Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. *Genome Res.* **24**, 1020-1027, doi:10.1101/gr.171264.113 (2014).

332 Ma, Y. *et al.* Increasing the efficiency of CRISPR/Cas9-mediated precise genome editing in rats by inhibiting NHEJ and using Cas9 protein. *RNA Biol* **13**, 605-612, doi:10.1080/15476286.2016.1185591 (2016).

333 Guenther, C. M. *et al.* Synthetic virology: engineering viruses for gene delivery. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* **6**, 548-558, doi:10.1002/wnan.1287 (2014).

334 Cronin, J., Zhang, X. Y. & Reiser, J. Altering the tropism of lentiviral vectors through pseudotyping. *Curr Gene Ther* **5**, 387-398 (2005).

335 Ho, M. L. *et al.* Efficiency of Protease-Activatable Virus Nanonodes Tuned Through Incorporation of Wild-Type Capsid Subunits. *Cellular and Molecular Bioengineering* **7**, 334-343, doi:10.1007/s12195-014-0334-y (2014).

336 Asuri, P. *et al.* Directed evolution of adeno-associated virus for enhanced gene delivery and gene targeting in human pluripotent stem cells. *Mol. Ther.* **20**, 329-338, doi:10.1038/mt.2011.255 (2012).

337 Hofherr, S. E., Mok, H., Gushiken, F. C., Lopez, J. A. & Barry, M. A. Polyethylene glycol modification of adenovirus reduces platelet activation, endothelial cell activation, and thrombocytopenia. *Hum. Gene Ther.* **18**, 837-848, doi:10.1089/hum.2007.0051 (2007).

338 Lee, G. K., Maheshri, N., Kaspar, B. & Schaffer, D. V. PEG conjugation moderately protects adeno-associated viral vectors against antibody neutralization. *Biotechnol. Bioeng.* **92**, 24-34, doi:10.1002/bit.20562 (2005).

339 Kay, M. A., He, C. Y. & Chen, Z. Y. A robust system for production of minicircle DNA vectors. *Nat. Biotechnol.* **28**, 1287-1289, doi:10.1038/nbt.1708 (2010).

340 Koo, T. *et al.* Selective disruption of an oncogenic mutant allele by CRISPR/Cas9 induces efficient tumor regression. *Nucleic Acids Res.*, doi:10.1093/nar/gkx490 (2017).

341 Lee, K. *et al.* Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. *Nat Biomed Eng* **1**, 889-901, doi:10.1038/s41551-017-0137-2 (2017).

**Figures legends**

**Figure 1 Genome editing platforms and mechanisms for DSB repair with endogenous DNA.**

Genome editing nucleases (ZFN, TALEN and CRISPR/Cas9) induce DSBs at targeted sites. DSBs can be repaired by NHEJ, or in the presence of donor template by HDR. Gene disruption by targeting the locus with NHEJ leads to formation of indels. When two DSBs target both sides of a pathogenic amplification or insertion, a therapeutic deletion of the intervening sequences can be created, leading to NHEJ gene correction. In the presence of a donor corrected HDR template, the HDR gene correction or gene addition induces DSB at the desired locus.

Abbreviations: DSB, double-stranded break; ZFN, zinc finger nucleases; TALEN, transcription activator-like effector nucleases; CRISPR/Cas9, clustered regularly interspaced short palindromic repeat associated 9 nucleases; NHEJ, nonhomologous end-joining; HDR, homology-directed repair.

**Figure 2 Ex vivo and in vivo genome editing for clinical therapy.**

Right: in ex vivo editing therapy, cells are isolated from a patient to be treated, edited and then re-engrafted back to the patient. \To achieve therapeutic success, the target cells must be able to survive in vitro and return to the target tissue after transplantation. Left: for in vivo editing therapy, engineered nucleases are delivered by viral or non-viral approaches, directly injected into the patient for systemic or targeted tissues (such as the eye, brain, or muscle) effect.

**Figure 3 Production of CAR T cell product with genome editing technology.**

**Figure 4 Viral and non-viral delivery systems for genome editing technology.**

The most commonly used viral vectors include adeno-associated viruses (AAV), lentiviruses and adenoviruses (AdV). Non-viral physical methods can be used for genome editing to deliver biomacromolecules intracellularly without the use of nanoparticles. Non-viral delivery may be microinjections in vitro, direct injection into the embryo or zygote ex vivo, or hydrodynamic injection in vivo. Alternatively, electroporation or mechanical deformation realize delivery by creating transient pores in the cellular membrane, making entries for genome editing biomacromolecules.

**Table 1 Comparison of ZFN, TALEN and CRISPR/Cas9 platforms**

|  |  |  |  |
| --- | --- | --- | --- |
|  | ZFN | TALEN | CRISPR/Cas9 |
| Recognition site | Zinc finger protein | RVD tandem repeat region of TALE protein | Single strand guide RNA |
| Modification pattern | Fok1 nuclease | Fok1 nuclease | Cas9 nuclease |
| Target sequence size | Typically 9~18 bp per ZFN monomer, 18~36 bp per ZFN pair | Typically 14–20 bp per TALEN monomer, 28~40 bp per TALEN pair | Typically 20 bp guide sequence + PAM sequence |
| Specificity | Tolerating small number of positional mismatches | Tolerating small number of positional mismatches | Tolerating positional/multiple consecutive mismatches |
| Targeting limitations | Difficult to target non-G-rich sites | 5ʹ targeted base must be a T for each TALEN monomer | Targeted site must precede a PAM sequence |
| Difficulties of engineering | Requiring substantial protein engineering | Requiring complex molecular cloning methods | Using standard cloning procedures and oligo synthesis |
| Difficulties of delivering | Relatively easy as small size of ZFN expression elements suitable for a variety of viral vectors | Difficult due to the large size of functional components | Moderate as the commonly used SpCas9 is large and may cause packaging problems for viral vectors such as AAV, but smaller orthologs exist. |

ZFN: Zinc finger nuclease; TALEN: Transcription activator-like effector nuclease; CRISPR: Clustered regularly interspaced short palindromic repeat.

**Table 2 Clinical trials of gene editing in the treatment of human diseases**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Platform | Disease applications | Target | Edited cells | Delivery | Sample size | Phase | Trial number |
| ZFN | HIV-1 infection | CCR5 | CD4+ T-cells | Adenovirus | 12 | 1 | NCT00842634 |
|  | HIV-1 infection | CCR5 | CD4+ T-cells | Adenovirus | 19 | 1 | NCT01044654 |
|  | HIV-1 infection | CCR5 | CD4+ T-cells | Adenovirus | 21 | 1/2 | NCT01252641 |
|  | HIV-1 infection | CCR5 | CD4/CD8 T-cells | Adenovirus | 26 | 1 | NCT01543152 |
|  | HIV-1 infection | CCR5 | CD4/CD8 T-cells | mRNA | 12 | 1/2 | NCT02225665 |
|  | HIV-1 infection | CCR5 | CD4+ T-cells | mRNA | 14 | 1 | NCT02388594 |
|  | HIV-1 infection | CCR5 | CD4+ T-cells | mRNA | 30 | 1/2 | NCT03666871 |
|  | HIV-1 infection | CCR5 | CD4+ T-cells | mRNA | 12 | 1 | NCT03617198 |
|  | HIV-1 infection | CCR5 | CD34+ HSPCs | mRNA | 18 | 1 | NCT02500849 |
|  | HPV-induced cervical precancerous lesions | HPV16/18 E7 | Epithelial cells | DNA | 20 | 1 | NCT02800369 |
|  | Mucopolysaccharidosis I | IDS gene | Hepatocytes | AAV | 9 | 1/2 | NCT03041324 |
|  | Mucopolysaccharidosis II | IDUA gene | Hepatocytes | AAV | 3 | 1/2 | NCT02702115 |
|  | Hemophilia B | Factor IX gene | Hepatocytes | AAV | 12 | 1 | NCT02695160 |
|  | β-thalassemia | BCL11A gene | CD34+ HSPCs | mRNA | 6 | 1/2 | NCT03432364 |
|  | Recurrent/Refractory malignant glioma | IL13Ralpha2 | CD8+ T cell | Injection | 6 | 1 | NCT01082926 |
| TALEN | Relapsed/Refractory B-ALL | CD52, TRAC | CAR T cells | Lentivirus | 18 | 1 | NCT02808442 |
|  | HPV-related cervical intraepithelial neoplasia | HPV16/18 E6/E7 | Epithelial cells | Plasmid | 40 | 1 | NCT03226470 |
| TALENs and CRISPR/Cas9 | HPV-related cervical intraepithelial neoplasia | HPV16/18 E6/E7 | Epithelial cells | Plasmid | 60 | 1 | NCT03057912 |
| CRISPR/Cas9 | AML | CD123, TRAC | CAR T cells | mRNA | 162 | 1 | NCT03190278 |
|  | Metastatic non-small cell lung cancer | PDCD1 | T cells | DNA | 12 | 1 | NCT02793856 |
|  | Castration resistant prostate cancer | PDCD1 | T cells | DNA | Withdrawn | 1 | NCT02867345 |
|  | Muscle-invasive bladder cancer | PDCD1 | T cells | DNA | Withdrawn | 1 | NCT02863913 |
|  | Advanced esophageal cancer | PDCD1 | T cells | DNA | 16 | 1 | NCT03081715 |
|  | Metastatic renal cell carcinoma | PDCD1 | T cells | DNA | Withdrawn | 1 | NCT02867332 |
| HIV-1 infection with ALL | CCR5 | CD34+ HSPCs | Liposome and electroporation | 5 | 1 | NCT03164135 |
|  | EBV positive cancers | PDCD1 | T cells | DNA | 20 | 1 | NCT03044743 |
|  | Relapsed refractory multiple myeloma, melanoma, synovial sarcoma, and myxoid/round cell liposarcoma | NY-ESO-1, TRAC PDCD1 | T cells | Lentiviral and electroporation | 18 | 1 | NCT03399448 |
|  | Relapsed or refractory CD19+ leukemia and lymphoma | TRAC, B2M | CAR T cells | Lentiviral and electroporation | 80 | 1/2 | NCT03166878 |
|  | Relapsed or refractory CD19- leukemia and lymphoma | CD19 and CD20 or CD22, TRAC | CAR T cells | Lentiviral and electroporation | 80 | 1/2 | NCT03398967 |
|  | Mesothelin positive multiple solid tumors. | PDCD1 and TRAC | CAR T cells | Lentiviral DNA | 10 | 1 | NCT03545815 |
| Mesothelin positive multiple solid tumors | PDCD1 and TRAC | CAR T cells | Lentiviral DNA | 10 | 1 | NCT03747965 |
|  | Metastatic gastrointestinal epithelial cancer | CISH | TIL | Electroporation | Withdrawn | 1/2 | NCT03538613 |
|  | T-cell leukemia or lymphoma | CD7, CD28 | CAR T cells | - | 21 | 1 | NCT03690011 |
|  | Neurofibromatosis type 1 | NF1 | iPSCs | DNA | 20 | 1 | NCT03332030 |
|  | β-thalassemia | HBB gene | iHSCs | - | 12 | 1 | NCT03728322 |
|  | β-thalassemia | BCL11A gene | CD34+ HSPCs | - | 45 | 1/2 | NCT03655678 |
|  | Sickle cell disease | BCL11A gene | CD34+ HSPCs | - | 45 | 1/2 | NCT03745287 |
|  | LCA10 | CEP290 gene | Photoreceptor cells | AAV | 18 | 1/2 | NCT03872479 |

ZFN: Zinc finger nuclease; CCR5: Chemokine receptor 5; HSPCs: Hematopoietic stem/progenitor cells; IDS : Iduronate 2-Sulfatase; IDUA: α-L-iduronidase; BCL11A: Mouse B-cell lymphoma factor 11A; B-ALL: B acute lymphoblastic leukemia; TRAC: T cell receptor alpha chain; TALEN: Transcription activator-like effector nuclease; CRISPR: Clustered regularly interspaced short palindromic repeat; AML: Acute myeloid leukemia; PDCD1: Programmed cell death 1; NF1: Neurofibromatosis type 1; TIL: Tumor-infiltrating lymphocytes; iPSCs: Induced progenitor stem cells; iHSCs: Induced hematopoietic stem cells; LCA10: Leber congenital amaurosis type 10；CEP290: Centrosomal protein 290; AAV: Adeno-associated virus.