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REVIEW ARTICLE

Application of CRISPR/Cas9 genome editing to the study and treatment of disease

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Abstract CRISPR/Cas is a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic elements. The CRISPR/Cas9 method has been engineered from the type II prokaryotic CRISPR system and uses a single-guide RNA to target the Cas9 nuclease to a specific genomic sequence. Cas9 induces double-stranded DNA breaks which are repaired either by imperfect non-homologous end joining to generate insertions or deletions (indels) or, if a repair template is provided, by homology-directed repair. Due to its specificity, simplicity and versatility, the CRISPR/Cas9 system has recently emerged as a powerful tool for genome engineering in various species. This technology can be used to investigate the function of a gene of interest or to correct gene mutations in cells via genome editing, paving the way for future gene therapy approaches. Improvements to the efficiency of CRISPR repair, in particular to increase the rate of gene correction and to reduce undesired off-target effects, and the development of more effective delivery methods will be required for its broad therapeutic application.

Keywords CRISPR · CRISPR/Cas9 · Genome editing · Inherited disease · Mutation correction · Gene therapy

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Introduction

Genome engineering is a field of research dedicated to the development of methodologies for targeted modification of the genome. This approach has the potential to revolutionize the study of disease mechanisms and to support the progress of personalized therapy for genetic disorders.

Historically (over 30 years ago), the manipulation of the genome has relied on homologous recombination (HR) (Folger et al. 1982) to modify specific genes in cells (Fig. 1). This was carried out by introducing a donor template with significant homology to the targeted region, and specific nucleotide changes were introduced in vitro in order to introduce the desired genetic mutation. HR was widely used in mice, however the low efficiency of HR in human cells, the need for screening/selection strategies and the potential for adverse mutagenic effects have limited its widespread implementation.

In the last decade, the pace of genome engineering, now coined "genome editing", has gained great momentum. Several new approaches have been developed including the use of zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs) and, more recently, two component RNA-guided endonucleases (RGENs), such as the clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein (Cas) (CRISPR/Cas) system, changing the landscape of genome editing in a wide variety of organisms (Zhang et al. 2014) (Fig. 1). These endonucleases generate double-strand breaks (DSBs) in the DNA. DSBs are most commonly repaired by the endogenous cellular DNA repair pathways of non-homologous end joining (NHEJ) or homology-directed repair (HDR), both of which can be used to edit the genome. NHEJ is an error-prone process that involves direct linking of the broken ends and can create disruptive

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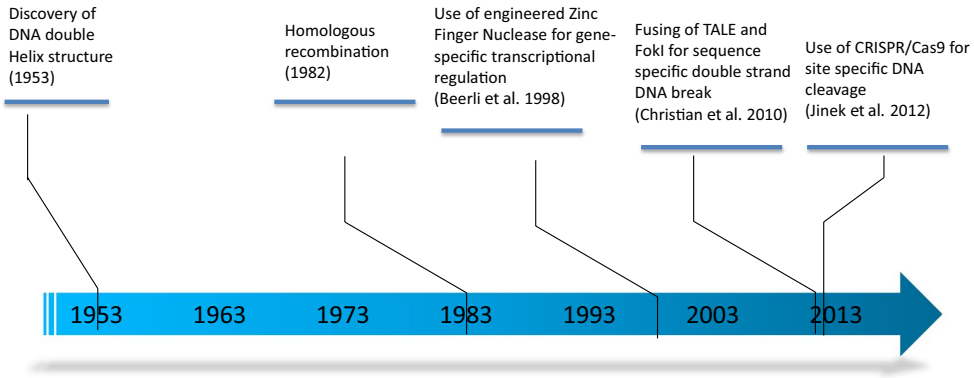


Fig. 1 Timeline of genome engineering research

insertions and deletions (indels) at targeted cleavage sites. The HDR pathway uses homologous DNA sequences as templates for repair and, by supplying an exogenous repair template, HDR can be exploited to precisely edit genomic sequence or insert exogenous DNA.

The CRISPR/Cas9 system has elevated genome editing from being a technical challenge to a practical reality due to the simplicity of the method. Unlike ZFNs and TALENs that use proteins to recognize specific sequences in the genomic regions, the CRISPR/Cas system uses a single guide RNA (sgRNA) that recognizes the genomic target via standard Watson–crick base pairing and acts as a scaffold for binding of the endonuclease Cas9. The ease of using a single RNA has led to the widespread adoption of sgRNAs for genome editing. The adaptability, simpler assembly, higher specificity and efficiency of CRISPR/Cas9 make it a very effective alternative to ZFNs and TALENs.

Scientists are now able to engineer any part of the human genome with extreme precision using CRISPR/Cas9 technology. This development has been hailed as a milestone in medical science since it has the potential to transform the study and treatment of a wide range of diseases, including inherited genetic disorders such as cystic fibrosis, viral disorders such as HIV, and cancer (Zhang et al. 2014; Wu et al. 2013; Doudna and Charpentier 2014).

In this review, we discuss how CRISPR/Cas9 genome editing is leading to significant progress in the study and treatment of disease, providing promise for advancements in human gene therapy.

Genome editing using CRISPR/Cas9

Origin of the CRISPR/Cas system

CRISPR and Cas genes function to provide adaptive immunity in bacteria and archaea against bacteriophage invading genetic material (Jinek et al. 2012). CRISPR repeats were first discovered in the *Escherichia coli* genome as an unusual repeat locus (Ishino et al. 1987). The significance of this structure was appreciated later when investigators realized that phage sequences are similar to the spacer sequences in CRISPR loci (Bolotin et al. 2005). Soon afterward, it was demonstrated that bacteria can acquire resistance against bacteriophages by integrating a genome fragment of the infectious phage into the CRISPR locus (Barrangou et al. 2007).

Adaptive immunity involves three phases: (1) adaptation phase, in which invading phage or plasmid DNA fragments are integrated into the CRISPR loci as "spacers"; (2) CRISPR RNA (crRNA) biogenesis phase, with transcription of precursor crRNAs, containing a repeat portion and an invader-targeting spacer portion, that then mature into crRNAs after cleavage within the repeat sequences; (3) invader silencing phase, in which crRNA-directed cleavage of foreign nucleic acid mediated by Cas proteins occurs (Doudna and Charpentier 2014).

There are at least eleven different CRISPR–Cas systems, which have been grouped into three major types (I–III) (Makarova et al. 2011b). The type II system from *Streptococcus pyogenes (S. pyogenes)* has been the most studied and has been engineered into an efficient genome-editing tool (Hsu Patrick et al. 2014). The advantage of the type II is that it only requires one Cas protein (Cas9) for nuclease activity and two other RNA components, the crRNA that guides the Cas to the complementary genomic target sequence and the trans-acting crRNA (tracrRNA), which is critical for crRNA maturation and for recruiting the Cas9 nuclease to the DNA (Jinek et al. 2012; Deltcheva et al. 2011).

Use of CRISPR/Cas9 for genome editing

The dual tracrRNA:crRNA has been engineered as a sgRNA containing two critical features: a DNA

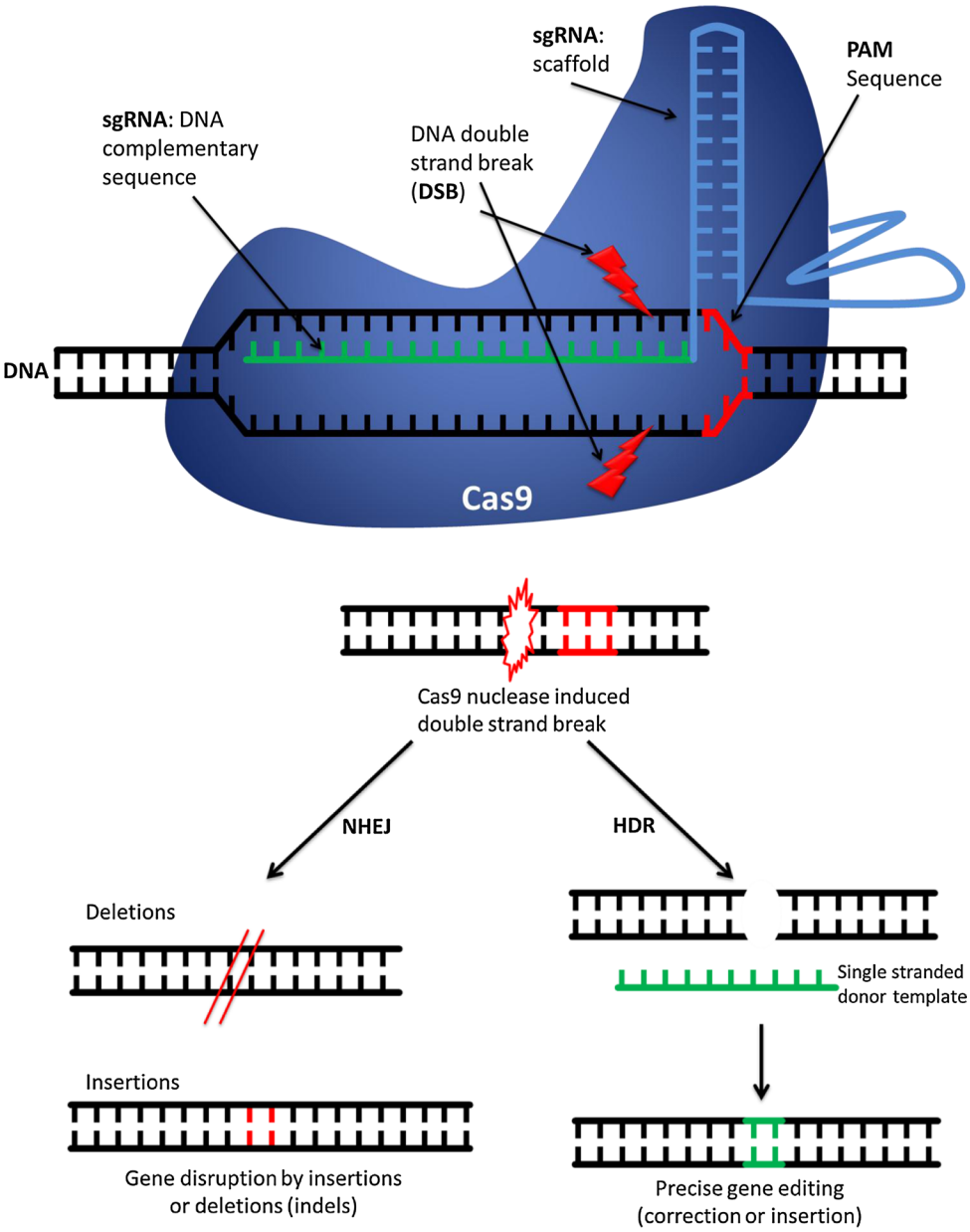


Fig. 2 Genome editing using CRISPR/Cas9 system. The Cas9 protein binds to the sgRNA scaffold and in the presence of the sgRNA complementary sequence and the PAM sequence it generates a double-stranded DNA break. The double-stranded DNA break triggers the endogenous cellular DNA repair machinery that catalyzes non-homologous end joining (NHEJ) or homology-directed repair (HDR)

complementary guide sequence (typically 20 nucleotides) and the double-stranded structure at the 3′ side of the guide sequence that binds Cas9 (Sternberg et al. 2014; Jinek et al. 2012; Gasiunas et al. 2012; Doudna and Charpentier 2014). The CRISPR/Cas9 technology uses the Cas9 protein to introduce site-specific DSBs in the target DNA sequences (Fig. 2). In order for the nuclease activity of Cas9 to occur, the CRISPR/Cas9 system requires a protospacer-adjacent motif (PAM) adjacent to the 3′ end of the DNA target site that facilitates the target recognition. PAM recognition is crucial in triggering the transition between Cas9 target binding and cleavage conformations (Sternberg et al. 2014). The PAM is also important as a recognition system control to protect the sgRNA itself from being targeted (Mali et al. 2013b). Thus, with this system, Cas9 nuclease activity can be directed to any genomic region that lies immediately 5′ of a PAM, simply by designing the guide sequence of the sgRNA so that it is complementary to the desired target DNA sequence. This is the most important advantage of the CRISPR/Cas9 system over ZFNs and TALENs systems that require protein engineering for each DNA target to be modified (Sander and Joung 2014).

Cas9-induced DSBs can be repaired by the endogenous DNA repair pathways present in all cell types and organisms: HDR and NHEJ (Davis and Maizels 2014). Both repair mechanisms are used by cells to maintain genomic integrity and cell viability. In the presence of a homologous repair template, the CRISPR/Cas9 system can be used to

generate precise sequence modifications (e.g., mutation insertion/correction) through HDR (Sander and Joung 2014). HDR frequently repairs DSBs during the S and G2 phases of the cell cycle. NHEJ can result in the introduction of insertion/deletion mutations (indels) that, if present in coding exons, can lead to a frame-shift and premature stop-codon formation, with disruption of the normal translational reading frame and inactivation of corresponding protein. This is the most predominant pathway of DSB repair in mammalian cells and it is not dependent on cell cycle (Mladenov and Iliakis 2011).

PAM

The PAM is a 3 base pair sequence that lies adjacent to the 3′ end of the DNA target site and facilitates the target recognition. The PAM sequence is specific to each Cas9 ortholog, even within the same species, and determines the DNA targeting locus of Cas9 (Hsu et al. 2013). The *S. Pyogenes* Cas9 is a versatile tool commonly used in genome editing, as it recognizes 5′-NGG and 5′-NAG PAM sequences (with the latter targeted with lower efficiency) that are found on average every eight base pairs within the genome (Jiang et al. 2013; Cong et al. 2013).

It is recognized that the formation of the RNA-DNA heteroduplex is initiated at the PAM site and that the binding of the PAM and the presence of a matching target DNA leads to Cas9 nuclease activity (Nishimasu et al. 2014). Imaging studies suggest that the complex composed of Cas9 and the RNA-DNA heteroduplex leads to Cas9 initiating DNA strand separation via an unknown mechanism (Sternberg et al. 2014) and subsequently leads to a DSB.

Cas9 protein function

The family of Cas9 proteins comprise DNA binding nucleases with heterogeneous lengths, ranging from 900 to 1600 amino acids (Makarova et al. 2002, 2011a), exclusively associated with the type II CRISPR system (Hsu et al. 2013). The Cas nuclease of the CRISPR system has an RNA binding domain, an α-helical recognition lobe (REC), a nuclease lobe that includes the RuvC and HNH nuclease domains for DNA cleavage, and a PAM-interacting site (van der Oost et al. 2014; Hsu et al. 2013; Hsu Patrick et al. 2014). The NHN domain of Cas9 is used to cleave the DNA strand complementary to the crRNA, and the RuvC domain is used to cleave the opposite DNA strand (Gasiunas et al. 2012).

A model has been proposed suggesting that Cas9 unbound to target DNA or guide RNA exhibits an autoinhibited conformation in which the NHN domain active site is blocked by the RuvC domain and is located far from the REC lobe (Jinek et al. 2014). In this way Cas9 can only cleave target DNA in the presence of a guide RNA that serves as a scaffold to allow the folding of the ribonucleoprotein (Hsu et al. 2013).

Off-target effects

Off-target mutagenesis is a concern for the application of CRISPR/Cas9 genome editing technology, especially with regard to gene therapy. Off-site targeting is defined by the tolerance of Cas9 to mismatches in the sgRNA sequence. It has been demonstrated that the target specificity depends on the design of the target site, the properties of the DNA-binding domain and the epigenetic status of the target site (Li et al. 2015).

Streptococcus pyogenes Cas9 specificity has been extensively studied by several groups that have created sgRNA variants containing one to four nucleotide mismatches in the complementary region in order to evaluate the abilities of the guides to direct Cas9 nuclease activity in human cells at reporter gene or endogenous gene target sites (Hsu et al. 2013; Fu et al. 2013; Cong et al. 2013). These studies suggest that mismatches are usually better tolerated at the 5′ end of the targeting region of the sgRNA compared to the 3′ end and are consistent with previous studies showing that the 8–12 bp at the 3′ end of the targeted sequence (named "seed sequence") are essential in the recognition phase (Semenova et al. 2011; Jinek et al. 2012; Jiang et al. 2013; Cong et al. 2013). Nevertheless, the effects of mismatches are not easily predictable, based only on their location within the sgRNA region. Other studies, in fact, demonstrated that Cas9 tolerates mismatches in a manner depending on their number, position and distribution (Pattanayak et al. 2013; Mali et al. 2013a; Hsu et al. 2013; Fu et al. 2013).

Potential off-target sites have been studied using computational methods that search genomic sequences with high similarity to the desired target locus. The use of whole-genome sequencing or other unbiased ways of labeling DSBs genome-wide may result in the identification of additional off-target sites. Various groups have provided algorithmic tools to predict the sequence of an optimal sgRNA with minimal off-target effects (e.g., http://tools.genome-engineering.org, http://zifit.partners.org and www.e-crispr.org) (Xie et al. 2014b; Xiao et al. 2014; Montague et al. 2014; Güell et al. 2014).

Despite extensive studies, it appears clear that for any given target site it is not currently possible to predict the number of tolerable mismatches, and to understand why only some sites are cleaved. In addition, the effect of the genomic/epigenomic context on the Cas9 nuclease activity is not clear. It seems possible that the chromatin structure can influence off-target site accessibility (Sander and Joung

2014), although some studies have not shown effects of DNA methylation on Cas9 activity (Hsu et al. 2013).

Off-target mutagenesis in human cells has been reported (Lin et al. 2014; Hsu et al. 2013; Fu et al. 2013), but recent whole-genome sequencing studies have suggested a high specificity of the CRISPR/Cas9 system with low incidence of off-target mutations (Smith et al. 2014; Suzuki et al. 2014; Veres et al. 2014).

Delivery methods

The CRISPR/Cas9 system has great potential for future applications in medicine; however, an efficient method is required to deliver the sgRNA- and Cas9-encoding cassettes to target cells in vivo.

RGENs have been delivered to a wide range of cell types and organisms using different delivery methods. Nuclease gene delivery through the cell membrane can be achieved by physical methods that have a number of advantages, such as simplicity, high reproducibility and enhanced gene expression (Ain et al. 2014). In cultured mammalian cells, electroporation (Ding et al. 2013), nucleofection (Mali et al. 2013c; Fu et al. 2013) and lipofectamine-mediated transfection (Mali et al. 2013c; Fu et al. 2013; Cong et al. 2013) of non-replicating plasmid DNA have been used to transiently express Cas9–sgRNAs complexes (Sander and Joung 2014). Recent experiments confirmed that the Cas9 protein-RNA complex can be introduced directly into cells using nucleofection or cell-penetrating peptides to enable rapid and timed editing (Ramakrishna et al. 2014; Kim et al. 2014). Transgenic organisms that express Cas9 from inducible promoters are also being tested (Doudna and Charpentier 2014).

Non-integrating viral vectors, including adenoviral, adeno-associated viral and lentiviral vectors have the advantage of not causing potential problems of insertional mutagenesis (Ain et al. 2014). It has been demonstrated that delivery with these vectors has varying levels of success, and recently RNA-guided CRISPR/Cas9 nuclease complexes have also been delivered using these methods (Maggio et al. 2014).

Transient expression of sgRNAs and Cas9 is usually sufficient to allow efficient genome editing. However, integrating lentiviral vectors have also been used to constitutively express Cas9 and/or sgRNAs in cultured human cells, showing consistent sgRNA and Cas9 expression with almost 100 % mutation rates at target sites (Wang et al. 2014; Shalem et al. 2014), as well as in mouse cells (Koike-Yusa et al. 2014). Constitutive expression could induce higher on-target editing efficiencies; however, persistent presence of RGENs in the cells might lead to increased off-target mutation rates, a problem previously reported using ZFNs (Gaj et al. 2012).

CRISPR-related RNAs and/or plasmid DNA have been delivered into embryos of zebrafish (Hwang et al. 2013), fruit flies (Yu et al. 2013; Gratz et al. 2013), mice (Zhou et al. 2014a; Li et al. 2013a) and rats (Li et al. 2013a) using microinjection. Plasmid DNA and RNA have also been injected into the gonads of adult roundworms (Waaijers et al. 2013; Katic and Großhans 2013; Friedland et al. 2013).

Optimization of the CRISPR/Cas9 system

Different approaches have been proposed to improve the efficiency and specificity of the CRISPR/Cas9 system.

One approach is the conversion of Cas9, which usually generates DSBs through the activity of its RuvC and NHN domains, in a DNA nickase that creates single-stranded breaks (SSBs), by catalytically inactivating one of the domains with point mutations (Sapranauskas et al. 2011; Jinek et al. 2012; Gasiunas et al. 2012). In this case, SSBs are repaired via the high-fidelity base excision repair pathway (Dianov and Hübscher 2013), and Cas9 can benefit from more precise NHEJ and HR (Hsu Patrick et al. 2014). A double-nicking strategy analogous to dimeric ZFNs or TALENs has been developed that uses a nickase variant of Cas9 with a pair of offset sgRNAs positioned on the target DNA that can mimic DSBs and mediate efficient indel formation (Shen et al. 2014; Ran et al. 2013; Mali et al. 2013a). As the off-target nick sites are perfectly repaired, there is an improvement of Cas9 specificity (Ran et al. 2013).

An alternative approach is to create fusions of the dimerization-dependent FokI nuclease domain (used in ZFNs and TALENs) to catalytically inactive versions of Cas9, called "dead" Cas9 or dCas9, that can be recruited to specific target sites by associated sgRNAs (Tsai et al. 2014; Guilinger et al. 2014). In this configuration, two fused dCas9-FokI monomers can simultaneously bind target sites at a defined distance with resulting cleavage by the FokI domains in the sequence between the two sgRNA target sites (Guilinger et al. 2014).

Another simple strategy to reduce off-target effects of Cas9 is to alter the length of the sgRNA, in particular the length of the targeting region of the sgRNA. Shorter sgRNAs truncated by two or three nucleotides at the 5′-targeting region can substantially reduce off-target effects without compromising on-target efficiency of modifications (Fu et al. 2014).

Enzymatic concentration is also crucial for Cas9 specificity. The amount of Cas9 enzyme expressed in the cells is an important factor in the tolerance to mismatches (Pattanayak et al. 2013). This appears particularly relevant as it has been reported that Cas9 can tolerate up to five mismatches within the target site (Fu et al. 2013). High concentrations

of the enzyme have been shown to increase off-target sites (Pattanayak et al. 2013; Hsu et al. 2013), giving higher off-target activity, while lower concentrations of Cas9 increase specificity and diminish on-target cleavage activity (Hsu et al. 2013). The duration of Cas9 expression also seems to be important as regards off-target activity (Mali et al. 2013b), but the mechanism underlying this phenomenon remains unclear and needs further investigation.

Application of CRISPR/Cas9 genome editing in disease

Use of CRISPR/Cas9 for the study of gene function

The CRISPR/Cas9 system offers a very efficient and highly versatile approach to produce gene knockouts for functional genomics studies in cells, providing an exciting new tool to dissect gene function in biological processes and disease (Zhang et al. 2014; Wu et al. 2013; Doudna and Charpentier 2014). Unlike other genome-editing techniques, such as ZFNs and TALENs, the target specificity of CRISPR/Cas9 is determined by the DNA complementary sequence of the sgRNA, enabling greater ease of construction of knockout reagents. The CRISPR/Cas9 system has been used to create knockout of the *CCR5* and *C4BPB* genes in human myeloid leukemia K562 cells, for example (Cho et al. 2013). Specific point mutations have also been introduced in mouse and zebrafish (Wang et al. 2013; Hwang et al. 2013; Heckl et al. 2014).

The molecular landscape of human cancer has been illuminated greatly over the past decade; however, the determination of which mutations are causally related to tumor initiation and progression remains incompletely understood. Importantly, it has been demonstrated that the CRISPR/Cas9 system can be used to model complex genetic diseases such as cancer as multiple mutations can be targeted at the same time using this technology. Cas9 can be used to target multiple genomic loci simultaneously by co-delivery of a combination of sgRNAs to the cells of interest (Zhou et al. 2014a; Wang et al. 2013; Li et al. 2013b). In an important advance, Sanchez-Rivera et al. (2014) have developed a novel CRISPR/Cas9-based approach for the rapid functional investigation of candidate genes in mouse models of cancer. Functional characterization of a range of tumor suppressor genes, including Pten and Apc (with known loss-of-function alterations in human lung cancer) was performed using a murine Kras(G12D)-driven lung cancer model. Cre-dependent somatic activation of oncogenic Kras(G12D) in combination with CRISPR/Cas9-mediated genome editing of the tumor suppressor genes resulted in lung adenocarcinomas with distinct histopathological and molecular features. Using another approach, Heckl et al. (2014) generated models of acute myeloid leukemia (AML) with cooperating mutations in genes encoding epigenetic modifiers, transcription factors and mediators of cytokine signaling (including Tet2, Runx1, Ezh2 and others), recapitulating the combinations of mutations found in patients. Through the delivery of CRISPR/Cas9 system using a lentiviral vector, loss-of-function mutations in up to five genes in a single mouse hematopoietic stem cell (HSC) were generated, resulting in clonal outgrowth and myeloid malignancy in the mice (Heckl et al. 2014). Thus, the CRISPR/Cas system allows the one-step generation of animals carrying mutations in multiple genes, an approach that enables the in vivo study of gene interactions, and allows for the engineering of a wide range of in vivo cancer models.

The CRISPR/Cas system has also been used to generate tumor-associated chromosomal translocations, which occur during carcinogenesis through illegitimate nonhomologous joining of two chromosomes. It is the ability of CRISPR/Cas9 to introduce DSBs at precisely defined positions that has enabled the generation of cancer cell lines and primary cells with chromosomal translocations that replicate those found in cancers, including Ewing's sarcoma, AML (Torres et al. 2014), and lung cancer (Choi and Meyerson 2014). Maddalo et al. (2014) have recently reported an efficient method to induce specific chromosomal rearrangements in vivo using viral-mediated delivery of the CRISPR/Cas9 system to somatic cells of adult mice. The EML4-ALK oncogene is present in a proportion of human non-small cell lung cancers (NSCLC), and CRISPR/Cas9 technology was used to create a mouse model of Eml4-Alk-driven lung cancer. The resulting tumors harbor the Eml4-Alk inversion, express the Eml4-Alk fusion gene, display histopathological and molecular features typical of ALK(+) human NSCLCs, and respond to treatment with ALK inhibitors.

Since the completion of the Human Genome Project, the functional characterization of all annotated genetic elements in normal biological processes and disease states is a primary goal. The ability of Cas9 to modify specific genomic loci provides a very efficient new tool to interrogate gene function on a genome-wide scale. Indeed several groups have recently determined the feasibility of using the CRISPR/Cas9 system to perform large-scale, loss-of-function screens in mammalian cells. Genome-wide lentiviral sgRNA libraries have been generated to allow for a pooled loss-of-function genetic screening approach that may be used for both positive and negative selection (Zhou et al. 2014b; Wang et al. 2014). For example, in the study by Wang et al. (2014), sgRNA expression cassettes were stably integrated into the genome, enabling a complex mutant pool to be tracked by massively parallel sequencing. A lentiviral library containing 73,000 sgRNAs was used to generate knockout collections, and screens were carried out in

the human leukemia cell lines HL60 and KBM7. Firstly, a screen for genes that function in the DNA mismatch repair pathway was successfully performed. Next, a screen for genes whose loss conferred resistance to etoposide, a chemotherapeutic agent that poisons the DNA topoisomerase II (TOP2A), identified TOP2A (as was expected) and in addition cyclin-dependent kinase 6 (CDK6). In order to identify genes essential for cell proliferation and other fundamental cellular processes, a screen was then performed for genes whose loss conferred a selective disadvantage on cells (negative selection) (Wang et al. 2014). This approach was also used to identify genes essential for cell viability in cancer and pluripotent stem cells in the study by Shalem et al. (2014). These, and other related studies, clearly illustrate the utility of CRISPR/Cas9 for carrying out large-scale genetic screens in mammalian cells and provide the foundations for a new era of functional genomics. While such studies have been previously attempted using RNA interference (RNAi) to reduce the expression of genes, this approach does not enable the generation of gene knockouts and confounding off-target effects to other mRNA can present a problem.

The unlimited self-renewing and multipotential differentiation ability of induced pluripotent stem cells (iPSCs) make them of particular value in disease modeling and gene therapy. Until recently, however, gene targeting in human (hiPSCs) had proven to be extremely difficult. Horri et al. (2013) recently created an iPS cell model for immunodeficiency, centromeric region instability, facial anomalies syndrome (ICF), a very rare autosomal recessive disorder, using CRISPR/Cas9 technology. ICR is caused by *DNMT3B* gene mutation, and in this study iPS cells were transfected with plasmids expressing Cas9 and sgRNA, disrupting the function of DNMT3B in transfected iPS cells. iPS cells with mutations in both alleles of *DNMT3B* were obtained in 63 % of transfected clones, and these cells were hypomethylated in satellite 2 repeats, which results in decondensation of these regions and centromeric instability, one of the hallmarks of this syndrome. This study demonstrates that the CRISPR/Cas9 system is highly efficient and useful for genome engineering of human iPS cells, and the generation of ICF syndrome model iPS cells will facilitate research of this rare condition (Horii et al. 2013).

Use of CRISPR/Cas9 for correction of gene mutations

Conceptually, the most direct approach to curing a human genetic disease is to correct the mutations that cause the disease through gene therapy. Indeed, the recent demonstration that the CRISPR/Cas9 system can be used for rapid and accurate genome editing in human stem cells in culture brings this possibility into sharp focus. In a recent ground-breaking study, CRISPR/Cas9 genome editing was successfully employed to correct the CFTR locus by homologous recombination in cultured intestinal stem cells of patients with cystic fibrosis (Schwank et al. 2013), a debilitating inherited disorder primarily affecting the lungs and digestive system. The corrected gene was expressed and fully functional; CRISPR/Cas9-mediated repair of the CFTR locus restored cAMP-induced intestinal stem cell organoid swelling, lost in CFTR mutant organoids of cystic fibrosis patients. This study demonstrates the feasibility of gene correction in primary adult stem cells derived from patients with a monogenic hereditary defect, and combined with a previous study, in which in vitro expanded organoids were transplanted into the colons of mice successfully (Yui et al. 2012), provides a potential strategy for future gene therapy in cystic fibrosis patients (Schwank et al. 2013).

β-Thalassemia, one of the most common genetic diseases, is caused by mutations in the human hemoglobin beta (HBB) gene. In another recent study, the combination of CRISPR/Cas9 to cleave the HBB gene and the piggyBac transposon (a mobile genetic element that efficiently transposes between vectors and chromosomes) to select for homologous recombination events, efficiently corrected two different β-thalassemia mutations and converted homozygous β-thalassemia to the heterozygous states in iPSCs from β-thalassemia patients (Xie et al. 2014a). No off-target effects were detected in the corrected iPSCs, and the cells retained full pluripotency and exhibited normal karyotypes. When differentiated into erythroblasts in culture, gene corrected iPSCs restored expression of HBB compared to the parental iPSCs line. These corrected iPSCs thus display normal function and potentially could provide a source of cells for transplantation in patients, offering a new strategy to cure this disease (Xie et al. 2014a).

Several recent studies have provided proof of principle for the CRISPR/Cas9 system to correct genetic disease in small mammal models. Using zygote injection of Cas9 mRNA and sgRNAs, mice or rats carrying desired mutations can be generated in one step. For example, Wu et al. (2013) have shown that mice with a dominant mutation in the *Crygc* gene that causes cataracts could be rescued by co-injection of Cas9 mRNA and a sgRNA targeting the mutant allele into zygotes. Gene correction was achieved via HDR based on an exogenously supplied oligonucleotide or the endogenous WT allele, and the resulting mice were fertile and could transmit the corrected allele to their progeny. One serious limitation to the therapeutic application of the CRISPR/Cas9 system is the potential for off-target mutations. The majority of the repaired mice (10 out of 12) showed no mutations at the ten potential off-target sites studied. In two mice, off-target mutations were detected at one of the potential off-target sites. These findings are consistent with other studies showing that injection of CRISPR/Cas9 into mouse and rat embryos results

in genetically modified small mammals carrying off-target mutations in rare instances only (Wang et al. 2013; Li et al. 2013a). Similarly, mice with mutations in the dystrophin gene (*Dmd*) that causes Duchenne muscular dystrophy were corrected using the same strategy of co-injection of Cas9 mRNA and a sgRNA targeting the mutant alleles into zygotes (Long et al. 2014).

Given that direct injection of CRISPR/Cas9 system into zygotes does not produce healthy progeny in all cases and can potentially generate off-target modifications, Wu et al. (2015) have recently explored the correction of genetic defects using this system in germline cells such as spermatogonial stem cells (SSCs). The group reasoned that CRISPR/Cas9 genome editing in SSCs containing a disease-causing mutation should allow for the selection of single SSCs carrying the required gene modification without other genomic changes. Such SSC lines could then be used to produce healthy offspring at 100 % efficiency. In this study, the CRISPR/Cas9 system was used to mutate an *EGFP* transgene or the endogenous *Crygc* gene in SCCs. The mutated SSCs proceeded with spermatogenesis following transplantation into the seminiferous tubules of infertile mouse testes. Round spermatids were generated and, after injection into mature oocytes, supported the production of heterozygous offspring showing the corresponding mutant phenotypes. Moreover, a deleterious mutation in *Crygc* ($Crygc^{-/-}$) pre-existing in the SSCs was repaired by CRISPR/Cas9-induced NHEJ or HDR, resulting in SSC lines carrying the corrected gene with no evidence of off-target modifications as shown by whole-genome sequencing. Fertilization using round spermatids generated from these lines produced offspring with the corrected phenotype at an efficiency of 100 % (Wu et al. 2015). These data demonstrate efficient gene editing in mouse SSCs via the CRISPR/Cas9 system and indicate promise in the use of this genome-editing system to treat genetic diseases through germline cell manipulation.

In a landmark study, Yin et al. (2014) achieved CRISPR/Cas9-mediated correction of a *Fah* mutation in hepatocytes in a mouse model of the human disease hereditary tyrosinemia, a rare condition resulting from the lack of a metabolic enzyme (FAH) required for tyrosine metabolism. Delivery of components of the CRISPR/Cas9 system by hydrodynamic tail vein injection resulted in initial expression of the wild-type Fah protein in a small proportion of liver cells (approximately 1 in 250). Drug induced expansion of Fah-positive hepatocytes rescued liver function and the body weight loss phenotype, thus demonstrating for the first time that genome editing with CRISPR/Cas9 was able to correct a disease mutation and phenotype in adult mice (Yin et al. 2014). Before this method can be applied in humans, several key challenges remain. Firstly, the correction frequency was very low initially at 0.4 % of the cells in the mouse liver, although the frequency of corrected cells increased to 33 % over time, sufficient to correct the weight loss defect with this disease. Most diseases do not lead to selection of corrected cells, however, and so it will be necessary to increase correction efficiency by improving delivery and activity of the CRISPR/Cas9 system. Secondly, the method used to deliver the CRISPR/Cas9 system to the mice, involving large-volume hydrodynamic injection, would require modification for humans.

Finally, Niu et al. (2014) have recently shown that precise gene targeting in cynomolgus monkeys could be achieved by co-injection of Cas9 mRNA and sgRNAs into one-cell-stage embryos, and no off-target mutagenesis was detected. This is an achievement of considerable significance since monkeys are a model species for studying human diseases and developing therapeutic approaches.

Further technological developments and conclusions

The CRISPR/Cas9 system enables rapid genome editing in different species at a very high efficiency and specificity, and is relatively easy to implement compared to other genetic-editing techniques (Sander and Joung 2014; Gupta and Musunuru 2014; Doudna and Charpentier 2014). The potential of this genome-editing method to illuminate gene function in biological processes and pathological states, and for the correction of gene defects in disease is great. Indeed, the successful correction by the CRISPR/Cas9 system of gene mutations in mouse models of human inherited disease and in primary adult stem cells derived from patients with monogenic hereditary defects may well lay the foundations for future gene therapy approaches (Zhang et al. 2014; Wu et al. 2013; Doudna and Charpentier 2014). It is widely recognized, however, that improvements to CRISPR repair efficiency and delivery methods will be required for its broad therapeutic application, in particular to increase the rate of gene correction and to reduce off-target effects (Zhang et al. 2014; Wu et al. 2013; Doudna and Charpentier 2014).

Arguably, the greatest block to the potential use of CRISPR/Cas9 technology in therapeutics is the fact that Cas9 can make cuts at off-target sites in the genome, and as a result has the potential to introduce mutations that are just as deleterious as the one being corrected (Sander and Joung 2014; Doudna and Charpentier 2014). Several groups are working to increase the specificity of the Cas9 enzyme in order to mitigate this effect. The use of the nickase variant of Cas9 and modification of the sgRNA construction for more accurate target recognition in the genome may enable researchers to increase specificity and to minimize undesired off-target effects (Gupta and Musunuru 2014; Cho

et al. 2014). Whole-genome analysis of off-target nuclease activity is also desirable, as it has been demonstrated that in silico predictions of Cas9 targets may present an incomplete picture of effects on the genome (Gabriel et al. 2011). Furthermore, the efficiency of genome-editing mechanisms is known to be dependent on cell type and cell cycle state, another important factor likely to impact genome-editing outcomes in vivo. Achieving a greater understanding of these issues is clearly important before applying this technology to the treatment of human disease. Recent genome-editing-based studies in animal models of inherited diseases demonstrates that the correction of only a modest number of cells can affect disease phenotype, offering hope for this approach, but it is recognized that the system would need to become many fold more efficient to have therapeutic potential in most settings.

DNA and RNA injection-based techniques are used for CRISPR/Cas9 delivery, such as injection of plasmids expressing Cas9 and the sgRNA and injection of CRISPR components as RNA. The development of improved or alternative delivery methods of the CRISPR/Cas9 system into cells in culture and organisms is essential for therapeutic application. These might include nanoparticles developed for nucleic acid delivery or viral vectors previously used successfully to treat genetic diseases of the liver with other genome-editing systems (Gaj et al. 2013).

Unsurprisingly, several commercial initiatives that seek to exploit the therapeutic potential of the CRISPR/Cas9 system are in development. These are welcome developments likely to increase the pace of technological advancement in CRISPR/Cas9 genome editing.

Genome-wide CRISPR/Cas9 knockout libraries have been developed with the potential to knockout each of the genes in the genome, allowing for a greater understanding of the role played by a given gene of interest in normal cellular and disease processes. CRISPR/Cas9 knockout libraries could potentially also be used to target regions of interest in the noncoding genome, such as promoters and enhancers. Moreover, the use of CRISPR/Cas9 for genome-wide studies (Wang et al. 2014) will enable large-scale screening for drug targets.

More controversially, it has been suggested that CRISPR/Cas9 genome editing could be used in the future to correct inherited mutations in IVF embryos, eliminating genetic diseases from affected families by changing the DNA of an embryo before implanting it into the womb. As mentioned above, the CRISPR/Cas9 system has been recently used to generate genetically modified monkeys (Niu et al. 2014), suggesting that there is no technical barrier to using genome-editing tools to modify human embryos. However, there are profound ethical issues concerning any genetic manipulation of human embryos and all safety concerns associated with the CRISPR/Cas9 method, most importantly off-target effects, would have to be fully addressed before this became an option. Moreover, as techniques such as pre-implantation genetic diagnosis can be used to select embryos that do not have the defective gene, it seems hard to justify any approach that involves genetic manipulation of the human embryo.

Despite the challenges, the huge advances in the field of gene therapy and genome editing by CRISPR/Cas9 achieved over the past few years pave the way for the development of an innovative and exciting new therapeutic strategy for the treatment of human inherited diseases, offering hope to patients.

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Conflict of interest The authors declare that there are no conflicts of interest.

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