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REVIEW ARTICLE

CRISPR-mediated genome editing and human diseases

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Abstract CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology has emerged as a powerful technology for genome editing and is now widely used in basic biomedical research to explore gene function. More recently, this technology has been increasingly applied to the study or treatment of human diseases, including Barth syndrome effects on the heart, Duchenne muscular dystrophy, hemophilia, β-Thalassemia, and cystic fibrosis. CRISPR/Cas9 (CRISPR-associated protein 9) genome editing has been used to correct disease-causing DNA mutations ranging from a single base pair to large deletions in model systems ranging from cells *in vitro* to animals *in vivo*. In addition to genetic diseases, CRISPR/Cas9 gene editing has also been applied in immunology-focused applications such as the targeting of C-C chemokine receptor type 5, the programmed death 1 gene, or the creation of chimeric antigen receptors in T cells for purposes such as the treatment of the acquired immune deficiency syndrome (AIDS) or promoting anti-tumor immunotherapy. Furthermore, this technology has been applied to the genetic manipulation of domesticated animals with the goal of producing biologic medical materials, including molecules, cells or organs, on a large scale. Finally, CRISPR/Cas9 has been teamed with induced pluripotent stem (iPS) cells to perform multiple tissue engineering tasks including the creation of disease models or the preparation of donor-specific tissues for transplantation. This review will explore the ways in which the use of CRISPR/Cas9 is opening new doors to the treatment of human diseases.
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Introduction

In 1987, Ishino et al discovered unusual DNA repeats of unknown function in the genome of the bacteria *Escherichia coli*.[1] Later, Mojica et al identified these same types of repeats in other microbes and termed these to be Clustered Regularly Interspaced Short Palindromic Repeats or CRISPR.[2–4] The CRISPR sequences were eventually found to act as an adaptive bacteria immune defense that destroyed viral pathogens by cutting the DNA of the invader with Cas nucleases.[5,6] Importantly, the Cas nucleases were made pathogen-specific by a unique property of the enzyme, which is the requirement for an RNA guide sequence that both activates the enzyme and selectively targets the nuclease to complementary DNA sequences. This unique property of the Cas nucleases have led to its application as a high-fidelity nuclease to produce DNA breaks or nicks at essentially any location desired in genomic DNA *in vivo*. Among the Cas nucleases, Cas9 from *E. coli* has become the most extensively studied and widely used. As a result of the DNA-sequence flexibility and specificity, the CRISPR/Cas9 RNA-guided DNA editing technology has been exploited in a rapidly growing number of basic science experimental studies involving mammalian and invertebrate systems.[7–11] While CRISPR/Cas9 is widely used in basic science research, the application of this technology in translational, disease-focused research is now emerging as an area of intensive investigation. This review will provide an overview of selected current research studies and also explore some of the future directions for the application of this technology in medicine.

CRISPR/Cas9 system

The gene editing process involves the generation of a double-stranded break (DSB) at the targeted DNA sequence. This DSB subsequently triggers two competing DNA repair systems which are homology-directed repair (HDR) or non-homologous end-joining (NHEJ, Fig. 1). NHEJ is an error-prone process in mammalian cells and can give rise to insertions or deletions (termed INDELs), either of which could change the protein coding sequences. In contrast, HDR involves homologous recombination with a donor DNA sequence to then introduce precise DNA mutations or the insertion of specific sequences in the targeted locus, such as the insertion of the DNA sequence encoding Green Fluorescence Protein (GFP).[12–15] The ability to use HDR to edit regions of the genome has prompted the development of multiple ways to selectively create DSBs in a sequence-specific manner. In total four major nuclease editing systems have been used to induce DSBs in the cell which include the zinc finger nucleases (ZFNs), the transcription activator-like effector (TALE)-nucleases (TALENs), the meganucleases, and most recently the CRISPR/Cas9 nuclease. The application of the CRISPR/Cas9 approach only requires designing the guide RNA (gRNA) sequence complementary to any desired target region to direct the Cas9 nuclease to this site. In contrast, the ZFNs, TALENs and meganucleases systems involve a time-consuming and costly procedure to generate a new protein specific for the individual target DNA sequence. Moreover, multiplex gene alterations are only really possible with the use of CRISPR/Cas9 because multiple gRNAs can be used simultaneously.[16–18]

The specificity of the CRISPR/Cas9 system is produced by the involvement of two essential components which are the Cas9 nuclease and the required gRNA (Fig. 1). The gRNA determines the specificity for a target DNA sequence through base-pair mediated binding to complementary DNA sequences. The binding of the gRNA then co-localizes Cas9 at the same specific-site, which leads to cuts in the DNA backbone and the generation of a DSB at the site.[19] Both the gRNA and Cas9 are introduced into cells by vectors created via the use of recombinant DNA technology, and depending on the application. These molecules can be expressed from either one or more different vectors.

Extensive experimental work has been used to both modify the Cas9 nuclease via the use of recombinant DNA technology or to identify Cas9 orthologues with different properties, such as changes in nuclease activity or in binding selectivity. The original form of Cas9 cleaves double-stranded DNA which triggers the DSB repair system in mammalian cells.[7] In contrast, the Cas9 mutant Cas9D10A, a CRISPR Nickase, was generated by Ran et al to selectively make single strand DNA cuts at the targeted DNA sequence.[20] Alternately, a nuclease-deficient Cas9, called "dead Cas9" or dCas9 was developed by Qi et al.[21] The dCas9 protein has no DNA cleavage activity and has been used to create fusion proteins that target either the promoter or other regulatory sequence of a gene with the goal of altering gene expression.[21–23] Furthermore, the Cas9 orthologues including CPF1 (CRISPR from Prevotella and Francisella 1),[24] the high-fidelity CRISPR-Cas9,[25] eSpCas9 ("enhanced specificity" *Streptococcus pyogenes* Cas9)[26] were subsequently applied in gene editing to improve the specificity of target site and reduce the cleavage of off-target effects.

Recently, the NgAgo (Natronobacterium gregoryi Argonaute) nuclease has been utilized for gene editing, and this nuclease is able to use single-stranded DNA as the guide sequence for targeting.[27] Furthermore, the NgAgo nuclease does not require a protospacer-adjacent motif sequence, which while short and common in the genome, still limits the target selection for the CRISPR/Cas9 system.

In the following sections, we summarize selected current studies using CRISPR/Cas9 as a novel therapeutic approach for human diseases utilizing both cell and animal models.

Cell models

CRISPR/Cas9 technology has been recently applied to disease-focused research through the production and characterization of patient-derived iPS cells from individuals with specific genetic diseases. The invention of induced pluripotent stem (iPS) cells has greatly advanced translational research, especially with the generation of disease-derived human iPS cells.[28,29] Strategies to reprogram somatic cells keep being updated, and the current

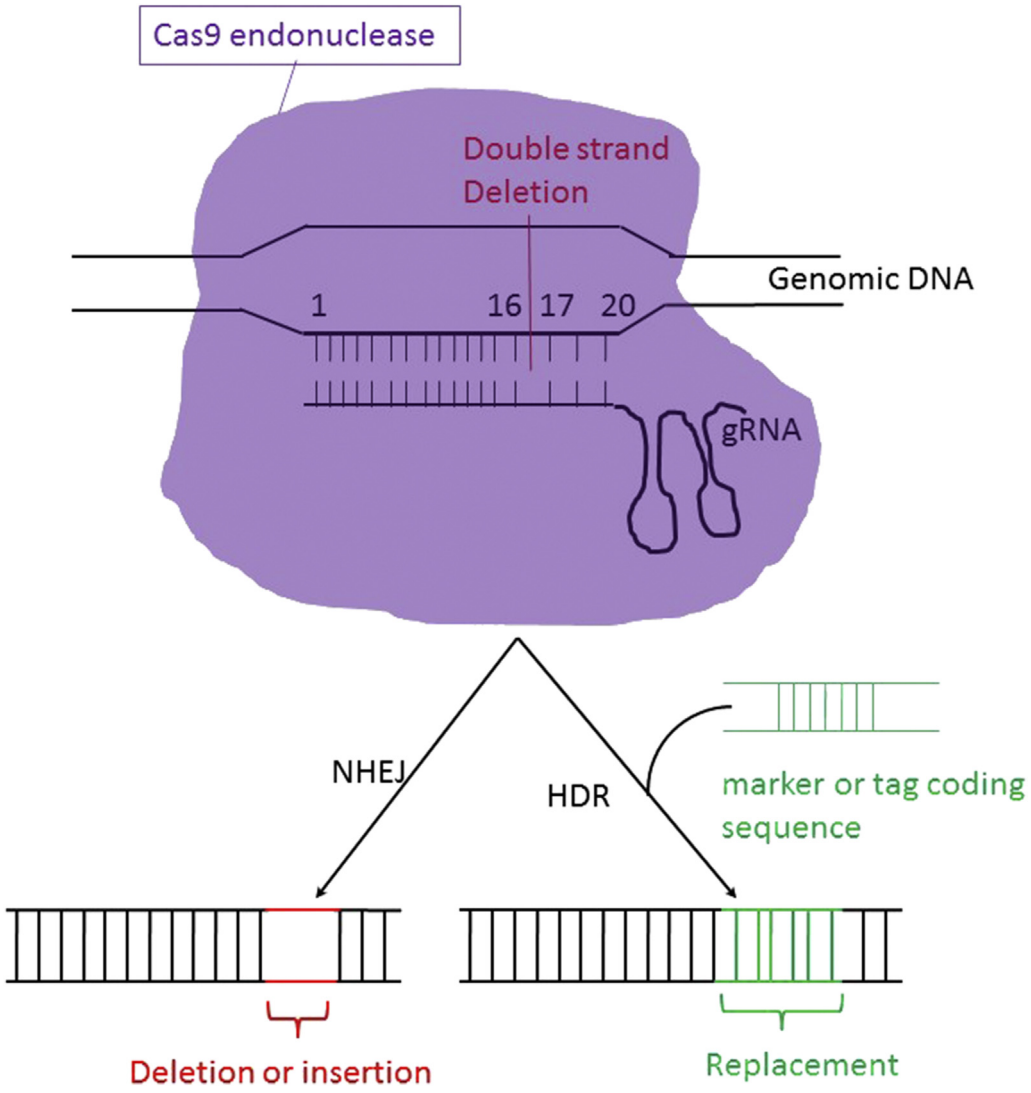

Fig. 1 CRISPR working mechanism. Guide RNA hybridizes with 20bp genomic DNA sequence and directs Cas9 endonuclease (colored in pink) to generate a double strand break which is usually located between 16 and 17bp region in the target sequence. Subsequently, DNA Mutagenesis is generated from DNA repair process, through either the non-homologous end-joining (NHEJ) or the homology-directed repair (HDR) mechanism. The final mutation could include insertion or deletion with several base pairs of DNA sequences (NHEJ pathway), or replacement with a particular DNA sequence used as a marker for further study (encoding for a fluorescence protein, tag protein, antibiotics, or the recognition sequence for a restriction enzyme digestion).

methods include using four Yamanaka factors (Oct4, Sox2, c-Myc and Klf4),[30] microRNA,[31] and small molecules/chemical compounds.[32–34] iPS cells have been generated and used as a "disease-in-a-dish" *in vitro* model for diseases including Duchenne and Becker muscular dystrophy, Parkinson's disease, Huntington's disease, and Down syndrome/trisomy 21.[28,35–37] These cells could be adapted to drug discovery, especially with the aid of high-throughput compound screening technology.[28,38–40] Additionally, enhanced DNA sequencing technologies can be used to identify the causal genetic mutation in an affected individual, and then gene editing with CRISPR/Cas9 can be used to demonstrate that the identified mutation is indeed directly responsible for disease phenotypes.

This strategy has been applied to the study of Barth syndrome, which is an X-linked genetic heart disease resulting from mutations affecting the *Tafazzin* gene. *Tafazzin* encodes a mitochondrial acyltransferase involved in the synthesis of the lipid cardiolipin, which plays critical roles in mitochondrial structure and function in the heart and other organs.[41] Wang et al generated the disease-specific iPS cells from Barth syndrome patients.[42] Then through a "heart-in-a-chip" model system, they discovered that genetic mutations affecting *Tafazzin* altered sarcomere assembly and cardiomyocyte contraction. The causal effect of these genetic mutations was also validated by creating a CRISPR engineered-*Tafazzin* mutation in iPS cells derived from healthy donors, which produced similar effects as those seen in the Barth syndrome-derived iPS cells. Furthermore, the iPS cells were used to test potential therapeutic agents for Barth syndrome. This pioneering study provided a framework for a "patient to patient" strategy to approach the study and potential treatment of human diseases (Fig. 2).

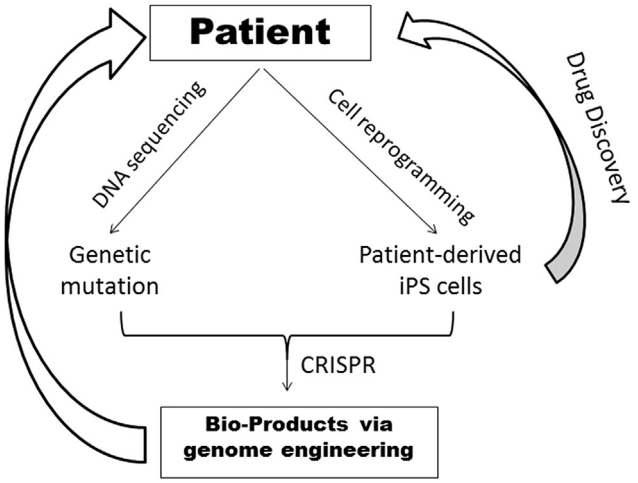

Fig. 2 CRISPR engineering for the clinics. Advances in DNA sequencing technology make it easier to identify the disease-driving genetic mutations. Meanwhile, the patient-derived iPS cells have been established to model human diseases and drug discovery *in vitro*. The deteriorating mutations can be corrected via the use of CRISPR-mediated gene editing, and the modified cells can be then utilized as patient-specific medicine.

An additional application of CRISPR/Cas9 modified iPS cells is through the use of tissues and organs derived from the iPS cells to provide personalized therapeutic transplants. This "patient to patient" workflow circumvents the issues of immune rejection after transplant as well as the problem of donor organ scarcity. The *in vivo* function of the CRISPR/Cas9-corrected iPS cells has been shown in mice with hemophilia by Park et al.[43] They transplanted differentiated endothelial cells created after the correction of factor VIII deficiency through the use of CRISPR/Cas9. The ability of the modified cells to be reintroduced into mice and correct the underlying disease clearly demonstrates the potential role of CRISPR/Cas9-modified iPS cells to be a potential cure for specific types of human diseases. More studies with CRISPR-modified disease-specific iPS cells are listed in Table 1 including the application to diseases produced by single base mutations, such as Barth syndrome,[42] β-Thalassemia,[45,46] and cystic fibrosis,[47] and to diseases resulting from deletions of larger DNA fragments, such as Duchenne muscular dystrophy[48] or specific types of hemophilia.[43]

CRISPR/Cas9 has also been applied to cancer immunotherapy where autologous T-cells can be engineered *in vitro* to express chimeric antigen receptors (CARs) that specifically recognize cancer cells. This approach to T-cell immunotherapy has proven to be promising in treating lymphoma,[49] leukemia[50,51] and melanoma in mice.[52] Similarly, Programmed Death 1 (PD-1), an inhibitory receptor in T-cells has become a therapeutic target. Blockade of the interaction between PD-1 and its ligand with an anti-PD-1 antibody has been clinically tested to suppress tumors[53,54] and this treatment was approved by The Food and Drug Administration in the US to treat melanoma.[53] Also the knockout of PD-1 in T cells via use of a Zinc finger nuclease[55] or genome editing by CRISPR was also tested as a strategy to boost T cells activity in the setting of tumor immunotherapy.[56]

Finally, the editing of specific T-cell expressed genes could be used to block the continual infection of T-cells in individuals infected with HIV. Specifically, the C-C chemokine receptor type 5, also known as CCR5, acts as a co-receptor for HIV and is essential for the infection of T-cells. Clinical trials have shown that the disruption of CCR5 via Zinc-finger nuclease-mediated genome editing in HIV patients is able to block the repeated cycles of infection and permits treated individuals to clear the virus.[57,58] Thus, genome editing opens a new avenue by which to modify critical receptors or other host proteins that are essential for the pathogenesis of infectious diseases.

Animal models

To model human diseases *in vivo*, scientists have been using CRISPR/Cas9 to generate genome-edited animals carrying genetic mutations responsible for a number of human diseases including mouse models of tyrosinemia[59] and lung cancer,[60] as well as rat[61] and monkey[62] models of muscular dystrophy. These models are useful for investigating disease pathology or the testing of candidate treatments. However, these animals can also be used to test the ability of CRISPR/Cas9-mediated gene editing to correct a disease-driving mutation *in vivo*. For example, Yin et al[59] tested whether the hydrodynamic injection of a single stranded DNA donor plus a Cas9 expression vector and sgRNA into the tail vein could cure the fatal genetic disease, type I tyrosinemia in the mouse. Type I tyrosinemia is due to mutations in the *Fah* gene which encodes the metabolic enzyme fumarylacetoacetate hydrolase, and the loss of this enzyme leads to the accumulation of toxic metabolites that kill hepatocytes. The authors found that CRISPR/Cas9 mediated gene editing led to expression of the wild-type *Fah* gene and also the survival and expansion of rescued hepatocytes. Importantly, the gene editing did not occur in all hepatocytes but instead the randomly edited hepatocytes were able to survive, grow, and then repopulate the liver. Hence, this study took advantage of a form of positive selection provided by successful gene editing, and may offer a new concept to effectively use this technology *in vivo*.

The CRISPR/Cas9 approach could also be potentially applied to the *in vivo* treatment of other genetic diseases. Duchenne muscular dystrophy (DMD), a fatal genetic muscle disease, is produced by in-frame deletions affecting the dystrophin gene.[63] Research laboratories of Charles A. Gersbach (Duke University),[64] Eric Olson[65] (University of Texas Southwestern Medical Center) and Amy Wagers[66] (Harvard Medical School) used an adeno-associated virus (AAV) to deliver the CRISPR/Cas9 editing system into the mouse with the goal of removing the deleterious DNA sequences and restoring the reading frame of *Dmd* gene in cardiomyocytes and muscle stem cells. This AAV-mediated CRISPR treatment was able to rescue muscle structure and function, and demonstrates the therapeutic potential of CRISPR in human diseases resulting from single-gene mutations.

In a related application of CRISPR/Cas9, the technique has been used to disrupt a crucial gene required for HIV integration into the host genome.[67,68] Specifically, the

Table 1 Studies using CRISPR modified disease-specific iPS cells.

Disease	Mutation	iPS	CRISPR	Function test	Reference
Chronic granulomatous disease	A single intronic mutation in the *CYBB* gene	Skin fibroblast	CRISPR/Cas9D10A, nickase; donor-mediated HR	*In vitro* differentiated macrophage function	Flynn et al, 2015, *Experimental Hematology*[44]
Barth syndrome	1 bp deletion or mutation	Skin fibroblast	CRISPR/CAS9 and PiggyBac, donor-mediated HR	*In vitro* differentiated cardiomyocyte; muscle contraction	Wang et al, 2014, *Nature Med*[42]
β-Thalassemia	C > T mutation in *HBB* gene	Fibroblast	CRISPR/CAS9 and PiggyBac, donor-mediated HR	*In vitro* hematopoietic differentiation; Gene expression	Xu et al, 2015, *Sci Report*[45]
β-Thalassemia	A/G and TCTT deletion in the *HBB* gene	Skin fibroblast	CRISPR/CAS9 and PiggyBac, donor-mediated HR	*In vitro* hematopoietic differentiation; Gene expression	Xie et al, 2014, *Genome Biology*[46]
Cystic fibrosis	CFTR F508 del	No iPS cells involved; 3D-intestinal organ cultures	CRISPR/CAS9, donor-mediated HR	*In vitro* differentiated intestinal organoids; Forskolin assay	Schwank, et al, 2013, *Cell Stem Cell*[47]
Duchenne muscular dystrophy	75484bp deletion including exon 44 of *Dystrophin* gene	Fibroblast	CRISPR/Cas9, donor-mediated HR	*In vitro* differentiated skeletal muscle cells; gene expression	Li et al, 2015, *Stem Cell Reports*[48]
Hemophilia A	Gene inversion (140 kb and 160 kb from intron 1 to 22)	Urine cells from hemophilia A patients	Cas9 protein and gRNA DNA plasmid were delivered by a microporator system.	*in vivo* differentiated endothelial cells; Transplantation into a hemophilia mouse	Park et al, 2015, *Cell Stem Cell*[43]

Khalili lab has delivered the CRISPR/Cas9 system via a tail-vein injection to target a HIV gene which is crucial for the integration of viral DNA into the host genome.[67] These treated animals demonstrated a reduced expression of HIV gene in multiple tissue organs, implicating a reduction in viral infectivity produced by CRISPR editing *in vivo*.

Sources of biologic therapies

Pigs organs including the heart, cornea, liver, and kidney, could become a new source of solid organs for transplant and provide a solution to the chronic shortage of available organs.[69] CRISPR makes it possible to simultaneously delete multiple genes, and this capacity sets CRISPR/Cas9 apart from other gene editing tools. The Church lab has used CRISPR/Cas9 to remove 62 retrovirus genes from the pig in order to obtain the retrovirus-free tissue organs that could be suitable for xenotransplantation.[70] The resulting tissue or organ replacement could prove useful in treating human diseases. For example, Elliott et al demonstrated that encapsulated pig islet cells can restore insulin production in patients with type 1 diabetes.[71] Additionally, there are other late-stage clinical trials testing the safety and effectiveness of pig to human transplants.[69]

CRISPR/Cas9 modified pigs have also been created to make products useful for biologic therapies. Human serum albumin (ALB) is therapeutically important for patients with liver failure and traumatic shock, however the high cost and low amounts available reduce its clinical use. As a result, transgenic pigs were developed as a source of human serum albumin, however the separation of the human albumin from the endogenous pig albumin presented a practical challenge. Zhang et al used CRISPR/Cas9-mediated gene editing to replace the pig albumin gene with the human albumin cDNA.[72] This created pigs that only produce recombinant human albumin, which provides a promising strategy to make other biomedical therapeutics, such as humanized polyclonal antibodies, in large domesticated animals.

Perspective

Genome editing in iPS cells or *in vitro* cultured cells holds great potential to treat human diseases, especially for

diseases produced by single-gene mutations. Patient-derived iPS cells can be tailored via CRISPR technology, selected *in vitro*, and delivered back to a patient to specifically replace defective cells or tissues (Fig. 2). Additionally, the pair of CRISPR/Cas9 with tissue engineering and regenerative medicine are paving the way to develop therapeutic biomaterials with either unique functional properties or on much larger scales than previously possible. However, there are both practical and ethical issues that currently represent potential barriers to the rapid application of this technology.

Since the CRISPR/Cas9 technology involves introducing vectors encoding both the gRNA and Cas9 into cells and tissues, a safe and efficient DNA delivery system are crucial to guarantee the success of gene editing. To address this challenge, various strategies are being developed to introduce the CRISPR/Cas9 components including the direct delivery of mRNA and protein or the design of new viral vector and non-viral vectors.[17,73–77] Anc80, an adeno-associated virus vector provides a good example of a new delivery system for *in vivo* gene editing and has been tested for multiple tissue organs including liver, muscle, and retina.[78] Other *in vivo* delivery systems such as microinjection and hydrodynamic transfection have been shown to be successful in animals. However, efficient and specific gene replacement *in vivo* is still challenging, and the ideal means to simultaneously deliver both CRISPR and the repair donor template into the desired tissues in the body remains to be developed.

Both critics and ethical concerns have concentrated on the application of CRISPR technology in humans.[79,80] Like the experience with stem cell research, it will take time for this novel technology to be accepted in medical practice and genuine safety and ethical issues that needs to be addressed as part of this process. To our excitement, UK parliament in 2015 approved three-parent mitochondrial therapy for women with severe mitochondrial diseases. Moreover, increasing number of CRISPR related clinical trials are being proposed worldwide.

Conflicts of interest

The authors declare no conflict of interest.

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