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CRISPR/Cas system: An emerging technology in stem cell research

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Abstract

The identification of new and even more precise technologies for modifying and manipulating the genome has been a challenge since the discovery of the DNA double helix. The ability to modify selectively specific genes provides a powerful tool for characterizing gene functions, performing gene therapy, correcting specific genetic mutations, eradicating diseases, engineering cells and organisms to achieve new and different functions and obtaining transgenic animals as models for studying specific diseases. Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology has recently revolutionized genome engineering. The application of this new technology to stem cell research allows disease models to be developed to explore new therapeutic tools. The possibility of translating new systems of molecular knowledge to clinical research is particularly appealing for addressing degenerative diseases. In this review, we describe several applications of CRISPR/Cas9 to stem cells related to degenerative diseases. In addition, we address the challenges and future perspectives regarding the use of CRISPR/Cas9 as an important technology in the medical sciences.

Key words: Gene editing; CRISPR/Cas9; Stem cells; Degenerative diseases

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Core tip: The possibility of translating new molecular knowledge systems to clinical research is particularly appealing for counteracting degenerative diseases as well as infective pathologies and cancer. A novel gene-editing technique, CRISPR/Cas9, has recently emerged for inducing targeted genetic modifications. Therefore, in this review, we describe recent applications of CRISPR/Cas9 to stem cells for counteracting

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INTRODUCTION

Gene editing

The development of gene targeting by homologous recombination (HR) was one of the fundamental steps forward in the field of genome editing, allowing site-directed specific mutation of a desired locus by exploiting homology arms to facilitate recombination at the donor site^[1]. HR-mediated gene targeting led to the generation of both knock-in and knock-out cell lines as well as many transgenic animal models. However, one of the weaknesses of this technology is that the frequency of recombination events is low (one in 10^6 - 10^9 cells)^[1], thus limiting its application for large-scale experiments. A subsequent fundamental discovery was the observation that targeted DNA double-strand breaks (DSBs) could directly induce homology-directed repair (HDR)^[2,3]. It was also shown that in the presence of a DSB without any homology repair template, the error-prone nonhomologous end-joining (NHEJ) repair pathway induces insertion or deletion mutations (indels) at the break site. These observations led to the development of programmable nuclease-based genome editing strategies based on the design of molecular machines composed of a specific DNA-binding domain and an effector domain to induce a DSB, thus increasing the rate of gene editing at the desired locus.

In particular, the zinc-finger nucleases (ZFNs; based on eukaryotic transcription factors)^[4] and the transcription activator-like nucleases (TALENs) from *Xanthomonas* bacteria^[5], which consist of individual modules targeting three or one nucleotides of DNA, respectively, can be assembled in different combinations and attached to the FokI nuclease domain to direct DSBs at a specific desired genomic site. Both types of proteins can be easily engineered due to the possibility of customizing the DNA-binding domain to recognize any sequence in the genome. A ZF consists of approximately 30 amino acids and can recognize 3 bp in the major groove of DNA. The possibility of developing synthetic arrays containing more than three zinc-finger domains allows the targeting of 9-18-bp-long DNA sequences, thus conferring enough targeting specificity within the human genome^[6]. A TALEN consists of a DNA-binding domain composed of a series of 33-35-amino acid modular repeats (each recognizing a single base pair) that are linked together to recognize contiguous DNA sequences. TALEN specificity is based on the exploitation of two hypervariable amino acids, known as repeat-variable di-residues^[7]. Compared to zinc-finger proteins, TALEN array engineering requires more technical work due to the extensive identical repeat sequences involved, but many strategies have been developed to overcome this issue.

ZFNs and TALENs applications

Both ZFNs and TALENs have been used to edit a number of genes and to introduce genome modifications. ZFN engineering has been applied to correct X-linked severe combined immune deficiency^[4], haemophilia B^[8] and sickle cell disease^[9,10]. ZFNs have also been applied for disease eradication *via* DSB-induced NHEJ, particularly in the field of acquired immune deficiency syndrome (AIDS). They were exploited to disable the human immunodeficiency virus 1 (HIV-1) co-receptor C-C chemokine receptor type 5 (CCR5), thus conferring virus resistance in T cells^[11] and haematopoietic stem cells^[12]; both approaches are currently in clinical trials. Another approach consists of the targeted integration of anti-HIV-1 restriction factors into the CCR5 locus to obtain T cells that are resistant to both CCR5-tropic (R5-tropic) and CXCR4-tropic HIV-1^[13]. The CCR5 deletion has twice been proven to be a powerful and effective way to eradicate HIV-1 from the human body. The first case dates back to a decade ago^[14]: the so-called "Berlin patient", who was receiving treatment with highly active antiretroviral therapy (HAART) after the diagnosis of HIV-1 infection, underwent two allogeneic haematopoietic stem cell transplantations from a donor with a homozygous mutation in the HIV-1 co-receptor CCR5 (CCR5 Δ 32/ Δ 32) to treat acute myeloid

leukaemia. The newly implanted cells no longer supported R5-tropic HIV-1 replication, and even after interruption of HAART, no active HIV-1 has since been detected in this patient. The second case, the so-called “London patient”, was actually very recent^[15]: An HIV-1-infected adult underwent allogeneic haematopoietic stem cell transplantation to treat Hodgkin’s lymphoma, again from a CCR5Δ32/Δ32 donor, but *via* a less aggressive and toxic approach, avoiding total body irradiation. At present, HIV-1 remission has been maintained in this patient. These two cases suggest that CCR5Δ32 bone marrow stem cell transplantation represents a possible strategy for achieving HIV-1 remission and should be deeply investigated in the future.

Similar to ZFNs, TALENs have been used to perform homologous recombination-based gene correction in induced pluripotent stem cells (iPSCs) from patients with β-thalassemia^[16]. TALENs were also exploited to induce point mutations in the *Oryza sativa* genome to obtain a new rice variety with enhanced resistance to herbicides^[17]. The first clinical application of TALENs consisted of a cell therapy approach based on the generation of universal chimeric antigen receptor 19 (CAR19) T cells by depletion of both TCR and CD52 molecules to eliminate the risk of graft-versus-host disease^[18].

However, the engineering of site-specific nucleases such as ZFNs and TALENs requires a great deal of effort, since the nucleases need to be *de novo* reengineered through a very labour-intensive and time-consuming procedure.

The CRISPR/Cas9 technology

A novel gene-editing technique, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, has recently emerged as an efficient alternative to ZFNs and TALENs for inducing targeted genetic modifications. The revolutionary feature of this technology is that Cas9 is an RNA-guided nuclease containing an HNH nuclease domain that cleaves the target strand of DNA and a RuvC-like nuclease domain that cleaves the non-target strand. Target sequence specificity arises from Watson-Crick base pairing between the guide RNA and the target DNA site^[19]. As a consequence, unlike previous strategies based on DNA-binding proteins, the CRISPR/Cas9 system can be easily programmed to target new sites by merely changing its guide RNA sequence, thus making it a suitable tool for high-throughput gene editing in many cell types and organisms.

The discovery of the CRISPR/Cas system originates in 1987, from mysterious 29-nt repetitive elements identified downstream of the *iap* gene in *E. coli*. Interestingly, these repeats were interspaced with five intervening 32-nt nonrepetitive sequences^[20]. During the following 10 years, the same pattern of repeated elements was reported in the genomes of different bacterial and archaeal strains, and in 2002 the acronym CRISPR was introduced to specify microbial genomic loci consisting of an interspaced repeat array^[21,22]. In parallel, a series of CRISPR-associated (*Cas*) genes adjacent to these repeat elements were identified^[22]. It was subsequently shown that CRISPR loci are actually transcribed^[23], and that bacteriophages are unable to infect archaeal cells carrying spacers corresponding to their own genomes^[24]. The first evidence that the CRISPR system serves as a microbial molecular immune memory and defence mechanism against viruses came from the Danisco company, where researchers were working to improve the lifespan of bacterial cultures for manufacturing yogurt and ice cream^[21]. Thus far, at least six types (I–VI, with types I–III the most characterized) of CRISPR/Cas systems have been identified in many Bacteria and in the majority of characterized Archaea; these systems consist of a cluster of CRISPR-associated (*Cas*) genes, noncoding RNAs and a distinct array of repetitive elements.

In general, a CRISPR system functions *via* three steps that are necessary to achieve a full immune response against foreign DNA^[25]. In the first stage, the invading DNA is fragmented into short sequences that are incorporated into the host crRNA array as spacers between the CRISPR RNA (crRNA) repeats. This stage is mediated by a complex of the Cas1 and Cas2 proteins, which are shared by all known CRISPR/Cas systems. In the second stage, the CRISPR array is transcribed into pre-crRNA, which is then cleaved and processed into mature crRNAs by Cas proteins and host factors^[26]. This crRNA acts as a guide containing the spacer sequence necessary to target specifically the Cas proteins to the invading genome upon recognition of the crRNA by the Cas proteins themselves. In particular, in type II CRISPR systems, the presence of a noncoding transactivating crRNA (tracrRNA) that hybridizes with the pre-crRNA is necessary for crRNA processing, Cas binding and target cleavage^[27]. crRNA maturation is mediated by either a Cas6-related ribonuclease (in type I and III systems) or housekeeping RNaseIII (type II system) that specifically cleaves double-stranded RNA hybrids of pre-crRNA and tracrRNA. In the third stage, the Cas proteins recognize the target DNA and induce cleavage of the invading genome, thus protecting the host cells from infection.

In the most recent classification, the various CRISPR/Cas systems are divided into two simple classes: class 1 CRISPR systems (types I, III, IV) utilize several Cas proteins

and crRNAs to form an effector complex, whereas class 2 CRISPR systems (types II, V, VI) exploit a large single-component Cas protein in conjunction with crRNAs to mediate interference^[28]. The type II CRISPR system is currently one of the best characterized, consisting of the Cas9 nuclease, a crRNA array that encodes guide RNAs and the required auxiliary tracrRNA, which helps to process the crRNA array into discrete units containing a 20-nt guide sequence and a partial direct repeat^[27]. Within the DNA target, each spacer is always associated with a protospacer-adjacent motif (PAM), which can vary depending on the specific CRISPR system^[29,30].

To simplify the system and make it utilizable for genome editing, the crRNA-tracrRNA duplex can be fused into a chimeric single guide RNA (sgRNA) and expressed in a plasmid under the control of the human U6 polymerase III promoter, whose only requirement for transcription initiation is the presence of a G nucleotide, which can eventually be added at the 5' end of the guide^[27,31]. A human codon-optimized version of Cas9 fused to the C-terminal SV40 nuclear localization signal has also been generated for the mammalian expression system^[31]. As a consequence, the Cas9-sgRNA complex can specifically target the DNA sequence that base pairs with the sgRNA and is adjacent to the PAM sequence and induce a DSB. Cas9 can therefore be targeted to any genomic locus only by customizing an approximately 20-nucleotide sequence complementary to the target DNA, making it an easily programmable platform for high-throughput gene targeting^[32].

Indeed, the CRISPR/Cas9 system has been used for both NHEJ- and HDR-induced gene editing in eukaryotic cells^[31,33-35]. Direct embryonic injection of sgRNA and Cas9 mRNA allowed transgenic mice with multiple modified alleles to be obtained^[36]. To improve the specificity of CRISPR/Cas9-mediated HDR, a nickase version of Cas9 (Cas9n) was generated by aspartate-to-alanine mutation in the RuvC catalytic domain to nick rather than cleave DNA, leading to a single-strand break^[19,27,37]. It has been reported that the combination of Cas9n together with a pair of offset sgRNAs complementary to opposite strands of the target DNA induces a double nick (one per DNA strand), leading to a DSB and NHEJ-based indels^[32]. Due to the combination of two sgRNAs, Cas9n shows fewer off-target effects than does Cas9, since possible individual single-stranded nicks are repaired by the high-fidelity base excision repair mechanism. Recently, the type V CRISPR/Cas system was discovered^[38], based on the Cpf1 ribonucleoprotein (CRISPR from *Prevotella* and *Francisella* 1), containing only the RuvC-like domain and not the HNH domain. In contrast to Cas9, Cpf1-mediated DNA cleavage is guided by only a crRNA and does not require a tracrRNA. Additionally, Cpf1 requires a short T-rich PAM preceding the target sequence, unlike the G-rich PAM downstream of the target sequence required for Cas9, and the seed region is within approximately the first five nucleotides at the 5' end of the target sequence.

Within the past few years, the RNA-targeting type VI CRISPR/Cas system was also discovered and characterized. This system is based on the Cas13 protein, which forms a crRNA-guided RNA-targeting effector complex when assembled with crRNA. The type VI CRISPR/Cas system can be divided into four subtypes (A-D) based on the phylogeny of the effector complexes^[39-41]. However, all type VI systems are based on Cas13, which exhibits two enzymatically distinct ribonuclease activities: One responsible for pre-crRNA processing and one provided by two Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domains, which are required for the degradation of the target RNA^[42-44]. These properties of Cas13 led to the rapid development of a new generation of RNA-targeting tools for many applications. In particular, Cas13 has been tested for human RNA knockdown, showing high specificity and fewer off-targets compared to RNAi^[45,46,51].

Source of human stem cells for genome editing

The possibility of combining the potential of human pluripotent stem cells (hPSCs) with this new genome-editing technique makes important applications in biomedical research possible. hPSCs can be generated either from human embryonic stem cells (hESCs), arising directly from embryos^[47], or from iPSCs. iPSCs are generated from fibroblasts or other somatic cells by the transfection of "reprogramming genes"^[48,49]. In addition, by transferring a nucleus from differentiated cells to a de-nucleated ovum, the third type of stem cell (SCNT stem cells) can be obtained^[50]. hPSCs generally share several characteristic features, such as the possibility of being maintained in culture for many passages with the same karyotype without genomic loss. hPSCs are pluripotent cells and can differentiate into different somatic cell types based on the protocol used^[51]. The ability of hPSCs to self-renew indefinitely and differentiate into different types of somatic cells represents an important tool for regenerative medicine. With this tool, mutations can be introduced in cell lines to generate disease models, and genetic defects can be corrected to rescue pathological conditions.

iPSC technology has provided appealing tools to the field of degenerative disease

research. iPSCs can be produced by injecting several key transcription factors into somatic cells. Initially, Takahashi *et al.*^[52] were able to reprogram murine fibroblast cells by injecting several transcription factors, such as octamer-binding transcription factor 4 (Oct4), sex-determining region Y-box 2 (Sox2), Krüppel-like factor 4 and cMyc. In particular, Oct4 prevents the expression of genes involved in the differentiation of ESCs and can reprogram somatic cells^[53,54]. In 2007, Yu *et al.*^[55] applied this technique to human somatic cells. Thus, human somatic cells can be reprogrammed to iPSCs by combining factors such as Oct4, Sox2, NANOG and LIN28^[52,55].

Moreover, this technology has been improved by using newly defined factors as well as different delivery systems. It has been demonstrated that in the absence of the Oct4 and Sox2 factors, genes involved in mesendodermal (*i.e.* GATA3, GATA6, and SOX7) and ectodermal commitment (*i.e.* SOX1, SOX3, and GMNN) can induce cell reprogramming^[56,57]. Additionally, the use of miRNAs such as miR-291-3p, miR-294, miR-295 and the miR-302/367 cluster, has been suggested for enhancing the reprogramming cells^[58,59]. In addition to fibroblasts, other kinds of cells can be induced to undergo reprogramming; the cells include B lymphocytes; neural progenitors; keratinocytes; cells arising from amniotic fluid, the liver, the stomach, or the pancreas; or cells harvested from blood or urine^[60].

Importantly, as iPSCs originate from the somatic cells of patients, they represent a specific source for transplantation therapy that prevents immunologic reactions. iPSCs have the same background as the patients from whom they are harvested. Because they carry the same genetic mutations as the patient, these cells provide a perfect disease model, which is important for understanding pathological conditions or identifying personalized therapeutic tools.

CRISPR/CAS9 APPLICATIONS IN DEGENERATIVE DISEASES

Haematological disorders

By improving the development of experimental models, CRISPR/Cas9 technology has contributed to a deep understanding of haematological disorders. The first haematological disorder to which CRISPR/Cas 9 was applied was sickle cell disease (SCD). SCD is caused by a single-nucleotide polymorphism in one of the haemoglobin genes and induces severe organ complications^[61,62]. Dewitt *et al.*^[61] corrected the mutation in CD34+ haematopoietic stem/progenitor cells (HSPCs). In particular, they delivered a ribonucleoprotein complex containing the Cas9 protein, an unmodified single guide RNA and a single-stranded DNA oligonucleotide donor to replace the mutation in HSPCs^[61]. When these cells were differentiated to erythroblasts, they produced low mRNA and protein levels of sickle haemoglobin and increased levels of wild-type haemoglobin. Also, when transplanted into mice, the cells maintained the edited gene for 16 wk and showed improved clinical characteristics^[61].

Recently, an *in vivo* model for studying myeloid malignancies by using CRISPR/Cas9 technology was proposed^[63]. Patients affected by these malignancies harbour three or five mutations contributing to a poor diagnosis. By using CRISPR/Cas9, researchers inactivated eight different alleles in a single HSPC; cells arising from this HPSC were able to induce leukaemia after transplantation in mice^[64]. Bejar *et al.*^[65] also used engineered CRISPR/Cas9 HSPCs carrying specific mutations to demonstrate that these cells are sensitive to azacitidine.

CRISPR and HIV

HIV-1 infection is currently treated with HAART, involving a combination of antiretroviral drugs that help to control viral load, thus delaying or preventing progression towards AIDS. This therapy does not eradicate the virus from the body, and it has to be continued throughout a patient's life. One of the main problems in achieving an effective HIV-1 cure is the persistence of latent viral reservoirs that cannot be cleared by current treatments. The establishment of these reservoirs is due to the integration of HIV-1 DNA into the cellular genome^[66], and the only way to eradicate them would be to delete directly or deactivate proviral DNA. To this end, it has been reported that the CRISPR/Cas9 system can be exploited to target and inactivate HIV-1 integrated DNA in Jurkat cells, resulting in no difference between active and inactive HIV-1 DNA transcription, suggesting a promising strategy for addressing latently infected cells^[67]. Other studies have demonstrated that it is possible to apply the CRISPR/Cas9 system to remove entirely the HIV-1 genome by using specific gRNAs directed at the long terminal repeats of the integrated HIV-1 genome in latently infected cells^[68-70]. The efficacy of adeno-associated virus (AAV)

vectors in the delivery of the CRISPR/Cas9 system into transgenic HIV-1-infected mice and rats through tail-vein injection to excise proviral DNA has also been shown^[69,71]. Additionally, the CRISPR/Cas9 system has been applied to reactivate the latent HIV-1 reservoir by using catalytically deficient Cas9-synergistic activation mediator technology^[72]. Zhang *et al.*^[73] showed that reactivation of the HIV-1 provirus was achieved in latently HIV-1-infected TZM-bl, Jurkat and CHME5 microglial cells, indicating the potential application of CRISPR/deficient Cas9-synergistic activation mediator as a “shock and kill” strategy to reactivate and induce cell death of latently HIV-1-infected cells.

Neurodegenerative diseases

Neurodegenerative diseases are severe pathological conditions with critical social outcomes. Unfortunately, the available therapeutic approaches are not able to treat effectively these degenerative disorders. In fact, the molecular and cellular defects causing neurodegeneration are not entirely understood, and specific therapeutic targets are lacking. Therefore, to identify the cellular and molecular pathways involved in neurodegenerative diseases, genetic screening performed by applying CRISPR technology has been proposed. Different targets involved in neurodegenerative diseases have been identified using CRISPR technology applied to human neurons obtained from iPSCs. Based on this strategy, Nakamoto *et al.*^[74] investigated the role of coenzyme Q10 in patients with multiple-system atrophy, a neurodegenerative disorder characterized by various combinations of neuronal dysfunction^[74]. Their findings demonstrated that a reduction in coenzyme Q10 levels, particularly in patients with COQ2 variants, contributes to neuronal apoptosis in patients affected by multiple-system atrophy, suggesting an effective therapy^[74]. The benefit of using CRISPR technology in studies related to Alzheimer’s disease (AD) is under debate because most AD cases are sporadic and have different causes. Mutations in the gene encoding amyloid precursor protein are found in a small percentage of patients (> 0.1%) even when overexpression of beta-amyloid peptide is detected in all AD patients^[75]. However, CRISPR technology can be useful for correcting autosomal-dominant mutations in presenilin 1 and presenilin 2 (PSEN2) that are found in the early onset AD^[76]. In fact, CRISPR/Cas9 has been employed to correct PSEN2 in iPSC neurons from a patient with a PSEN2N141I mutation^[77].

The APOE4 isoform is involved in the development of late-onset AD^[78]. In contrast, the APOE2 isoform seems to reduce the risk of developing AD by up to 40%. Therefore, the application of CRISPR/Cas9 to replace APOE4 with APOE2 may be considered a useful tool for treating patients carrying the APOE4 variant^[79].

Huntington’s disease (HD) is characterized by muscular, psychiatric and cognitive disorders due to heterozygous expanded (CAG)_n trinucleotide repeats in the gene that encodes huntingtin (HTT). This disorder causes alteration of the medium spiny neurons. Cellular strategies have been suggested for the generation of an HD disease model and the identification of therapeutic tools for treating HD. Therefore, therapies based on stem cell transplantation have been indicated as promising therapeutic tools^[80]. In addition, iPSC lines originating from patients with juvenile HD have been generated^[81]. In this context, the application of the CRISPR technique to target the HTT locus in iPSCs has given rise to new perspectives for the treatment of HD^[82].

Bone and musculoskeletal disorders

The application of CRISPR technology to iPSCs originating from patients with skeletal disorders has been suggested to explore bone diseases. This approach has been applied to investigate cleidocranial dysplasia (CCD), a skeletal disease caused by a mutation in the transcription factor RUNX2. In particular, the CRISPR/Cas9 system has been applied to two iPSC lines generated from CCD patients with different RUNX2 mutations to restore the normal phenotype^[83]. The CRISPR-edited cells were then evaluated *in vitro* and in a rat model, and correct osteo-induction was observed, thus indicating the molecular mechanism involved and suggesting a novel therapeutic approach for treating CCD^[83]. The most abundant non-collagenous protein found in bone is osteocalcin, and an *in vivo* osteocalcin deficiency model shows impaired skeletal structure^[84]. To understand better the role of osteocalcin in skeletal disorders, a rat model was generated by Lambert *et al.*^[85]. Specifically, these researchers injected CRISPR/Cas9 to knock out osteocalcin in the pronuclei of Sprague-Dawley embryos. With the development of this system, the authors provided a model of the disease that can be used in the field of osteoporosis and osteoarthritis research.

Duchenne muscular dystrophy (DMD) is a severe disease that affects skeletal and cardiac muscles in childhood. The absence of the dystrophin protein, encoded by the dystrophin gene (*Dmd*), prevents the muscular sarcolemma from being protected from injuries due to contractions, causing DMD^[86]. Mutations in the *Dmd* gene are frequent, among which frameshift mutations are the most common, although in-

frame and out-of frame mutations may also occur; frameshift mutations generally result in a premature stop codon by altering the reading frame^[87]. Therefore, as DMD is a genetic disorder, the possibility of identifying a therapeutic approach for DMD based on the application of CRISPR/Cas9 technology to stem cells appears intriguing.

To repair damaged muscle by correcting the dystrophin gene, CRISPR/Cas9 has been applied in mdx mice, a model of DMD^[86]. By using this technique, the researchers obtained genetically mosaic animals with heterogeneous percentages of DMD gene correction (from 2% to 100%). These different percentages of gene correction allowed comparison of the percentage of correction with the level of muscular rescue^[86]. Interestingly, the dystrophin protein has also been restored in iPSCs obtained from patients affected by DMD by using CRISPR/Cas9 technology^[88]. However, five off-target sites were affected by the procedure in this model^[88].

Musculoskeletal disorders also occur in lysosomal storage diseases (LSDs). LSDs include different genetic diseases characterized by deleterious mutations causing the disruption of lysosomal enzymes. Therapeutic approaches for counteracting LSDs include enzyme replacement therapy, pharmacological chaperone therapy and haematopoietic stem cell transplantation. However, all of these treatments cause secondary side effects^[89]. To identify new therapeutic approaches, experimental models using CRISPR/Cas9 and iPSCs have been adopted. Pompe disease is an LSD caused by mutations in the gene that encodes the lysosomal hydrolase acid-alpha glucosidase and is characterized by a severe myopathy^[90]. Possible therapy for these patients is provided by the enzyme replacement therapy Myozyme[®]; unfortunately, this therapy is expensive. Therefore, a useful therapeutic approach involving targeting the mutation in hematopoietic stem cells (HSCs) derived from the same patient *via* the CRISPR/Cas9 system has been suggested^[91]. Other LSDs can certainly also be considered prospective targets for this therapy based on the CRISPR/Cas9- and iPSC system.

Cardiovascular diseases

CRISPR/Cas9 editing has emerged as a useful technology in the cardiovascular field. Cardiovascular disorders affect a large number of patients, and the incidence of these pathologies has increased considerably in recent decades. Therefore, an important challenge is to understand the molecular mechanisms that affect vascular and cardiac systems and determine cardiovascular mortality^[92]. Among the pathological conditions affecting the cardiovascular system, cardiomyopathies, arrhythmias, rheumatic heart disease, stroke and congenital cardiac defects have been reported. Molecular tests and bioinformatics analyses allow the identification of individuals predisposed to cardiac disorders. However, there are some limitations to a complete understanding of the molecular signalling causing these pathologies because mechanistic studies aimed at understanding the causes of the diseases are limited by the complexity of culturing human cardiomyocytes^[93]. However, CRISPR/Cas9 technology has allowed cardiac disease models to be generated, and it is possible to study cardiovascular diseases by injecting the CRISPR/Cas9 system components into the embryos of rats, rabbits and primates^[94,95]. In addition, the coupling of iPSC technology with the application of the CRISPR/Cas9 system has provided useful cell models for better understanding the molecular mechanisms involved in cardiac pathologies and for recovering specific mutations causing cardiovascular diseases. The combination of iPSCs and CRISPR/Cas9 technologies has allowed the generation of a cellular model characterized by mitochondrial dysfunction originating from patients affected by Barth syndrome. By introducing a mutation in the *tafazzin* gene with the CRISPR/Cas9 system, the authors demonstrated that this mutation caused the mitochondrial phenotype and that normal mitochondrial function could be recovered by the administration of specific antioxidants^[96].

CRISPR/Cas9 technology has also allowed the analysis of titin gene mutations in cardiomyopathy. By introducing either missense or frameshift mutations in the titin gene, researchers were able to generate contractile deficits in iPSCs that differentiated into cardiomyocytes (iPSC-CM)^[97]. Similarly, iPSC-CMs have been obtained from patients affected by Jervell and Lange-Nielsen syndrome, a severe cardiac arrhythmia^[98], and iPSCs carrying a mutation in the *CALM2* gene reproducing long QT syndrome have been generated with the same technique^[99]. As CRISPR/Cas9 may introduce changes in noncoding regions, Beaudoin *et al*^[100] were able to delete a sequence in an intronic region in the *PHACTR1* gene (associated with premature myocardial infarction) in iPSCs to generate a cell model of the pathology.

Hypertrophic cardiomyopathy is a severe cardiovascular disease with different clinical aspects characterized by cardiac arrhythmias. To identify therapeutic strategies for rescuing arrhythmias, hPSCs-MC have been engineered by using CRISPR/Cas9. In particular, Mosqueira *et al*^[101] generated in three hPSC lines carrying 11 variants of the c.C9123T-MYH7 mutation, which affects the myosin heavy chain to

cause hypertrophic cardiomyopathy. By using this disease model, the authors demonstrated the possibility of correcting arrhythmias by pharmacological treatment and identified the ratio between MHY7: MYH6 and mutant: wild-type MYH7 isoforms as a diagnostic tool^[101].

Diabetes

Stem cell therapy has been proposed for the treatment of diabetes, a metabolic disorder characterized by the disruption of insulin production. Two different types of diabetes are known: type 1 diabetes (T1D), which is an autoimmune disease, and type 2 diabetes (T2D), which is the most common and heterogeneous form of diabetes^[102]. Both T1D and T2D are characterized by the disruption of pancreatic β -cell function^[102].

The generation of pancreatic cells followed by their transplantation in patients with T1DM has been proposed. In this context, the use of iPSCs and the concurrent application of CRISPR/Cas9 technology can improve the generation of pancreatic organs^[103]. In addition, this system avoids the controversial use of hESCs. Despite the advantages of using hESCs, such as the ease of differentiating these cells into β cells *in vivo*, the reduction in viral transgene incorporation and the greater efficiency of these cells in producing insulin compared to iPSCs, ethical concerns due to the induction process restrict their use^[103].

T2D pathophysiology is complex because various factors, such as genetic, epigenetic and lifestyle factors, can contribute to the development of this disease. iPSC lines generated from T2D patients have allowed the detection of several mutations in transcription factors involved in pancreas development (*HNF1B*, *HNF4A* and *HNF1A*), genes encoding enzymes related to insulin secretion and proteins devoted to exocrine pancreas function^[104]. Interestingly, genome-wide association studies (GWAS) revealed a robust statistical association between T2D and genetic variants located in noncoding regions. Therefore, in association with GWAS, CRISPR/Cas9 has been suggested to be a useful tool for improved understanding of the molecular factors involved in the pathogenesis of T2D^[105]. A form of diabetes caused by mutations in the gene encoding insulin can appear during neonatal life (neonatal diabetes)^[106]. Recently, Balboa *et al*^[106] demonstrated that insulin mutations cause abnormal β -cell differentiation in a neonatal diabetes model. In particular, the researchers obtained iPSCs from affected patients. Then, by applying the CRISPR/Cas9 system, they corrected a missense mutation in the insulin gene and compared these corrected iPSCs to mutant iPSCs. Interestingly, by single-cell RNA sequencing, these authors observed increased endoplasmic reticulum stress and reduced proliferation^[106] in mutant cells compared to corrected cells.

Cancer

iPSCs can be generated from cancer cells. Therefore, this technology will allow the molecular bases of malignant transformation to be identified. In addition, this approach can result in the screening of therapeutic formulations and the identification of useful biomarkers. The generation of iPSCs *via* the application of CRISPR/Cas9 methodology is particularly important to identify genetic disruptions that induce cellular transformation and have not yet been found, *e.g.*, in the case of glioblastoma (GBM). GBMs belong to the gliomas, a heterogeneous type of cancer, and originate from cells showing neural stem and progenitor cell characteristics^[107]. Even though GWAS have allowed the identification of many genetic and epigenetic targets, other key molecular targets still need to be identified. For example, the *PKMYT1* gene has been identified as a candidate target for therapy in GBM patients by the application of CRISPR/Cas9 libraries to stem cell-like cells originating from GBM patients^[108].

T cell-based immunotherapy represents a useful tool for the treatment of malignant cells. These cells show a reduced proliferative ability, but the possibility of using iPSCs from antigen-specific T cells overcomes this limit. Unfortunately, the rearrangement of the T cell receptor chain gene during reprogramming causes loss of their antigen specificity. However, Minagawa *et al*^[109] were able to prevent this additional rearrangement by obtaining functional iPSCs from antigen-specific T cells *via* the application of CRISPR.

In the context of precision oncology, the application of CRISPR/Cas9 combined with iPSC technology offers effective tools for identifying appropriate therapies. Recently, this system allowed the investigation of the individual roles of two co-recurrent genetic lesions involved in myeloid malignancy: A mutation in the SRSF2 factor and a chromosome 7q deletion^[110]. The authors found that the SRSF2 mutation induces dysplasia, whereas the chromosome 7 deletion prevents differentiation and is associated with disease progression^[110].

The use of CRISPR/Cas9 technology associated with iPSC generation has been applied to the study of RET mutations in multiple endocrine neoplasm type 2 (MEN2). MEN2 is a rare syndrome that affects organs originating from neural crest

and endoderm and causes medullary thyroid cancer, pheochromocytoma, cutaneous lichen amyloidosis and primary hyperparathyroidism. In addition, it can cause Hirschprung disease^[111]. iPSCs from a MEN2 patient with the most frequent mutation in RET (RET^{C634Y}) have been used to better understand the molecular mechanism by which the RET mutation causes MEN2^[112]. These researchers generated CRISPR-corrected isogenic counterparts of these cells and, by performing transcriptomic analyses, identified early growth response 1 as a key molecular target in MEN2A^[112].

In addition to the work described above, many other studies related to the application of the CRISPR/Cas9 System in stem cell research have been recently performed (Table 1).

CHALLENGES

As previously described, CRISPR/Cas9 has become a powerful technology that allows the manipulation of almost any biological organism. The relative simplicity of the technique has made it possible to develop new models for studying the effect of mutations in genetic diseases and for revealing previously unknown gene functions, among many other applications.

Despite the enormous therapeutic potential of the technique, it will be necessary to address various challenges before it can be safely used in the field of gene therapy and in clinical applications.

Off-target

The specificity of CRISPR/Cas9 is fundamental for its clinical application. Off-target mutations can impair the fitness and/or the functionality of edited cells and, even more problematically, can generate potential oncogenic cell clones^[113].

Initial reports of the whole-genome sequencing of edited cells indicate a low rate of off-target mutations, supporting the good specificity of the system^[114-116]. A study published in 2017 raised concerns about the extent of unexpected mutations introduced by Cas9^[117], but the study was retracted in 2018 due to insufficient data to support the claim^[118].

Subsequent studies based on whole-genome sequencing addressed concerns about potential off-target effects, reporting no unexpected off-target activity of CRISPR/Cas9^[119,120]. Another study indicated that by appropriately designing gRNAs, it is possible to achieve efficient *in vivo* editing with no detectable off-target mutations^[121].

A recent study revealed that sgRNAs are very sensitive to chromatin state, suggesting that off-target effects are inhibited by chromatin, thus favouring specificity^[122].

Overall, CRISPR/Cas9 appears to be a very specific tool for genome editing, and the initial discordant reports might have been more closely related to the appropriate choice of sgRNAs, rather than to Cas9 activity^[113]. Similar to PCR protocols, it is possible to envision that in the future, when sufficient data are available, a database of optimal sgRNAs can be generated to be used in different cellular models, paired with improved computational analysis, for gene editing.

Despite these reassuring data, new methods are being developed to detect potential off-target CRISPR mutations, as well as new systems and protocols to reduce further the risk. These approaches include the development of better *in silico* computational prediction tools, the use of more-specific nucleases, such as Cpf1, and the development of cell-free genomic DNA assays to detect double-stranded breaks based on sequencing, such as Digenome-seq^[123] (in which Cas9 cleavage is followed by next-generation sequencing) and newer, more-sensitive methods such as CIRCLE-Seq and SITE-Seq. Additional methods are being developed using cell-based assays and are aimed at identifying potential off-target sites in specific cell types; these methods include GUIDE-Seq^[124] and LAM-HTGTS^[125], the latter of which is aimed at identifying genomic rearrangements following DSBs^[126].

It has been reported that high concentrations of Cas9 nucleases may increase the rate of off-target mutations^[127,128]. To address this issue, new strategies such as the double nickase system^[92] or the use of high-fidelity recombinant Cas9 variants have been developed^[128-131]. In addition, the discovery and characterization of Cas9 orthologues from other prokaryotic organisms may help to identify Cas variants with higher specificity^[132,133].

Recently, protocols based on the transfection of Cas9-coding mRNA and gRNA as well as gRNA-Cas9 complexes have been proposed as systems to reduce further off-target effects^[134]. The delivery of CRISPR/Cas9 components as RNA and gRNA-Cas9 complexes may present an additional advantage, since circular plasmid DNA may

Table 1 Recent studies related to the gene-editing technology applied to stem cells research on degenerative diseases

Authors and year	Disorder
Zhou <i>et al</i> ^[163] , 2018	Spinal muscular atrophy
Calvo-Garrido <i>et al</i> ^[164] , 2019	Neuronal
Dong <i>et al</i> ^[165] , 2019	Hereditary hearing loss
Zhao <i>et al</i> ^[166] , 2019	Breast cancer
Yanagihara <i>et al</i> ^[167] , 2019	Skeletal diseases
Vrugt <i>et al</i> ^[168] , 2019	Fanconi anemia
Blanas <i>et al</i> ^[169] , 2019	Colorectal cancer
Sun <i>et al</i> ^[170] , 2019	Glioblastoma
Jelinkova <i>et al</i> ^[171] , 2019	Duchenne muscular dystrophy
Hurtado <i>et al</i> ^[172] , 2018	Renal
Tang <i>et al</i> ^[173] , 2019	Cardiac hypertrophy
Tian <i>et al</i> ^[174] , 2019	Pediatric biliary atresia
Wang <i>et al</i> ^[175] , 2018	Werner syndrome
Barnes <i>et al</i> ^[176] , 2018	Neuronal
Frasier <i>et al</i> ^[177] , 2018	Cardiac arrhythmia
Sasaki-Honda <i>et al</i> ^[178] , 2018	facioscapulohumeral muscular dystrophy
Wang <i>et al</i> ^[179] , 2018	Hepatoma
Moghaddas <i>et al</i> ^[180] , 2018	Autoinflammatory
Liu <i>et al</i> ^[181] , 2018	Colon cancer
Jiao <i>et al</i> ^[182] , 2018	Cardiac disorders
Lyu <i>et al</i> ^[183] , 2018	Haemophilia
Deng <i>et al</i> ^[184] , 2018	Retinitis pigmentosa
Wattanapanitch <i>et al</i> ^[185] , 2018	Thalassemia
Suda <i>et al</i> ^[186] , 2018	Parkinson's disease

(presumably only rarely) be randomly integrated into the host genome^[135].

Cellular challenges

The editing of a specific gene sequence relies on HDR rather than NHEJ. HDR is selectively expressed during mitosis and is downregulated after cell division^[136]. For this reason, gene editing may be very difficult to achieve in non-dividing cells, such as neurons. Different strategies are currently under study to address this issue^[137].

In vivo delivery challenges

Some genetic diseases may be treated by collecting, modifying and reinfusing stem cells, but others will require the correction of many cells in formed tissues in the patient's body.

An *ex vivo* strategy based on the collection of stem cells from a patient (usually from bone marrow), followed by their modification and reimplantation, presents almost the same general risks previously described for genome editing in cell cultures. Additional challenges clearly remain concerning the *in vivo* delivery of the CRISPR/Cas9 system. Lentiviral vectors have been widely and successfully used in different applications.

However, permanent integration of lentiviral vectors in the host cell genome will most likely cause permanent expression of the Cas9 nuclease, increasing the potential for off-target effects *in vivo* and the related oncogenic risk, which adds to the intrinsic risk of random insertion of these vectors in the cell genome.

Unlike lentiviral vectors, adenoviral (AV) vectors do not integrate into the host cell genome, thus avoiding permanent expression and reducing the risk of off-target effects. AV vectors also allow the insertion of larger DNA fragments, making it possible to include additional sgRNA sequences or reporter genes. On the other hand, AV vectors present risks of immunotoxicity due to cellular immune responses, and studies are consequently needed to define the immunogenicity of Cas9 for *in vivo* applications^[138].

AAV vectors have been proposed as more suitable and less risky viral vectors, and these vectors have been approved for use in clinical trials^[139]. Problems due to the

small genome size of AAV vectors have been addressed by using a smaller Cas9 variant from *Streptococcus aureus*, *Streptococcus thermophilus*^[37] or *Neisseria meningitidis*^[37], rather than the commonly used *Streptococcus pyogenes* Cas9 (SpCas9)^[140].

Even if AAV vectors are used, the problem of the persistent Cas9 expression remains, as do the potential risks of a lower editing efficiency due to previous immunity against AAV.

Delivery to embryos to generate knock-out or other mutants is possible through direct microinjection, which is a costly and technically challenging procedure, although it is useful in generating permanent germline modifications. This approach is the most common tool used by researchers to generate new animal models.

Recently, different approaches based on the development of non-viral vectors have been developed. Such delivery alternatives involve the use of lipid-based vectors, polymeric cationic vectors and chitosan^[141].

These methods are characterized by lower immunogenicity and higher safety, reducing the risk of short and long-term adverse effects. However, a low delivery efficiency remains the principal problem^[141]. Studies in the field of nanotechnology will most likely result in new, optimized synthetic delivery systems based on nanoparticles that will facilitate the delivery of CRISPR/Cas9 components *in vivo*.

Immunity against Cas9

Other issues that will need to be addressed include the risk of an immune response against Cas9, a prokaryotic protein, when used in gene therapy applications and how this may impact the application of the technique in a clinical context^[142].

A recent study^[143] of human donors documented a high frequency of antibodies and anti-Cas9 cytotoxic T-lymphocytes (CTLs) against SaCas9 and SpCas9; these Cas9 orthologues are the most widely used and are derived from *Staphylococcus aureus* and *Streptococcus pyogenes*, respectively. Since these are two common bacterial species infecting humans, the study raises concerns about the impact of pre-existing humoral and cellular immune responses to Cas9 in future clinical trials. A possible solution may be to use Cas9 orthologues derived from bacterial species that do not commonly infect humans, to avoid the destruction of cells “treated” using CRISPR/Cas9 due to pre-existing anti-Cas9 cellular immunity.

HIV resistance

In the field of AIDS, the advantage of CRISPR/Cas9 engineering consists of conferring permanent protection against HIV-1, which is not achieved with antiviral drugs, but an important unanswered question is whether and how HIV-1 might escape from this genome editing system. HIV-1 evolution experiments have been performed in CD4+ T cells expressing both Cas9 and sgRNAs targeting different regions of the HIV-1 genome^[144,145], showing that although there was apparent initial virus inhibition, viral replication re-bounded over time, resulting in high levels of HIV-1 production. In particular, rapid escape was observed when non-conserved HIV-1 sequences were deleted, while a longer time was needed to escape in the case of more conserved sequences. When the targeted viral DNA was sequenced, mutations were specifically identified in the sgRNA complementarity region, suggesting that HIV-1 can adapt its genome to escape CRISPR/Cas9-mediated editing. In particular, most of the identified resistance mutations were indels matching the specific site at which Cas9 was expected to cleave viral DNA, suggesting that a variety of mutations at the cleavage site might actually be induced by NHEJ: some of these mutations would not be selected because of abolishing viral replication, while other mutations would be selected because they are not deleterious to the virus, thus generating CRISPR/Cas9-resistant viral particles^[145].

To overcome this unique viral escape mechanism, one solution may be to exploit multiple sgRNAs to target conserved proviral regions. It has been shown that multiplexed targeting of HIV-1 DNA leads to much stronger suppression of HIV-1 infection, although possible viral escape cannot be excluded^[146]. Another approach might involve modified versions of Cas9 that can cleave the DNA outside of the target sequence, so that any mutation generated by NHEJ will not prevent the CRISPR/Cas9 machinery from rebinding and cleaving proviral DNA again. The newly discovered Cpf1 that cleaves DNA in the more distal region of the target sequence^[38] may provide a possible strategy for addressing this issue. Another solution could be to suppress the NHEJ machinery enzymes through the use of specific anticancer drugs^[147].

Other possible strategies for the suppression of viral infections are based on targeting host cell factors necessary for HIV-1 replication, such as inactivation of the co-receptor genes CXCR4 and CCR5. Several studies have already demonstrated the feasible application of CRISPR/Cas9 to inactivate both receptors^[148-150], thus generating HIV-1 resistant cells. CXCR4 or CCR5 knock-out T cells have also been produced by

direct electroporation of the CRISPR/Cas9 ribonucleoproteins^[151], which is a particularly useful strategy for cells that are difficult to transfect, such as primary cells. The immediate activity of the proteins is observed following transfection, and this approach may limit off-target effects, since the protein complex is quickly degraded within the cell.

Despite the promising efficacy of CRISPR/Cas9 for genome editing, the procedure is still too unsafe to be applied in human embryos because unwanted germline mutations might be passed to future generations, with unpredictable effects.

The first trial was carried out by Chinese researchers who used the CRISPR/Cas9 system to modify genetically the human β -globulin gene, whose mutation causes β -thalassemia, in human embryos^[152]. Unfortunately, a higher frequency of mutations was detected in the CRISPR/Cas9-treated human embryos compared to the results observed in modified adult mouse or human cells. This result confirmed that the fidelity and specificity of the CRISPR/Cas9 system still require further investigation, which will be a prerequisite for any clinical applications of genome editing. Despite these ethical concerns, the first genetically modified babies were recently reported to have been generated in China, giving rise to strong international criticism^[153]. He Jiankui, a genome-editing researcher at the Southern University of Science and Technology of China in Shenzhen, injected the CRISPR/Cas9 machinery into human embryos to disable the *CCR5* gene, thus generating R5-tropic HIV-1-resistant human babies. When the embryos were 3-5 five days old, a few cells were removed and checked for editing. Sixteen of 22 embryos were actually found to have been edited, and 11 of them were used in six implantation attempts before a twin pregnancy was achieved. Genetic tests suggest that both *CCR5* alleles had been correctly modified in one twin, while the other twin is heterozygous for the modification. At present, this type of gene editing is prohibited in most countries, as the CRISPR/Cas9 technology is still experimental. The rate of off-target mutations is still too high, which might lead to long-term unexpected side effects, including the development of cancers that may be passed to future generations. Furthermore, *CCR5* depletion provides higher susceptibility to other viral infections, such as West Nile and influenza viruses, and if a working vaccine against HIV-1 is found in the future, harbouring the *CCR5* deletion will provide no benefits^[154].

P53 mutations

hPSCs are very difficult to treat using CRISPR/Cas9 and exhibit a very low efficiency of genome editing compared to laboratory tumour cell lines^[155]. These characteristics are due to the toxicity of DSBs induced by Cas9 in hPSCs, which appear to be p53 dependent^[155]. Since stem cells may acquire p53 mutations^[156], clonal expansion of stem cells that are more tolerant to DNA damage poses severe risks of cancer development. A careful genetic analysis of hPSC-treated cells, therefore, needs to be carried out before clinical use.

Nature spread

Homing gene drives based on CRISPR/Cas9 may be used to design mutations that will spread within a target population or species, for instance, to confer resistance to a parasite^[157]. Such drives have been studied as a potential tool for the eradication of mosquitos to prevent diseases such as malaria^[158,159] or other vector-borne diseases. This possibility, though fascinating, raises many concerns, since it may potentially cause the genetic modification of an entire species if modified organisms are accidentally released in the environment. Safeguarding strategies are under development to avoid the risk of premature release in the wild^[157].

Ethical concerns

The technique needs to be used carefully and responsibly. Where does a cure end and improvement start?

A committee of the National Academy of Science addressed clinical, social, ethical and legal issues linked to genome editing, releasing a report entitled "Human Genome Editing: Science, Ethics, and Governance" in 2017^[160]. Permanently editing germlines raises many concerns^[161,162]. While there is no doubt that the correction of a genetic defect may help to eradicate, or at least significantly reduce, the burden of severe genetic diseases in the general population, the technical shortcomings of the technique will necessitate the discarding of embryos or even recurrent selective abortion when the editing procedure does not succeed, raising ethical, religious and practical concerns when applied to humans.

In addition, it may be difficult to distinguish between the correction of a detrimental mutation and genetic enhancement. For this reason, the use of genome editing technologies in human embryos may result in unexpected, unpredictable and potentially harmful consequences for future generations, since it may result in

reduced human genetic variability and cross the borders of eugenics, baby design and the removal of certain characteristics, to be substituted with others that are more desirable^[161,162].

Thus, the boundaries between a cure and eugenics applications are becoming very thin. It may be fundamental to promote general, worldwide-accepted protocols, which will require close interaction between the regulatory agencies, scientific communities and governments of different countries. It is for this reason that a global moratorium on the use of genome editing technologies for human germline modification has recently been called for^[154], to allow time to discuss the relevant scientific and ethical issues.

In conclusion, the potential of CRISPR/Cas9 is enormous, but researchers need to proceed with caution. It is very likely that new discoveries, data and protocols will help to address the many obstacles involved, and CRISPR will lead to a new revolution in the field of molecular biology, similar to polymerase chain reaction in the 1980s.

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