ISSN 1948-0210 (online)

World Journal of *Stem Cells*

World J Stem Cells 2019 November 26; 11(11): 904-1019

Published by Baishideng Publishing Group Inc

WJSC

World Journal of Stem Cells

Contents

Monthly Volume 11 Number 11 November 26, 2019

REVIEW

MINIREVIEWS

ORIGINAL ARTICLE

Basic Study

SYSTEMATIC REVIEW

World Journal of Stem Cells
Volume 11 Number 11 November 26, 2019

Contents

NAME OF JOURNAL
World Journal of Stem Cells

ISSN
ISSN 1948-0210 (online)

LAUNCH DATE
December 31, 2009

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Tong Cao, Shengwen Calvin Li, Carlo Ventura

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1948-0210/editorialboard.htm

EDITORIAL OFFICE
Jin-Lei Wang, Director

PUBLICATION DATE
November 26, 2019

INSTRUCTIONS TO AUTHORS
https://www.wjgnet.com/bpg/gerinfo/204

GUIDELINES FOR ETHICS DOCUMENTS
https://www.wjgnet.com/bpg/GerInfo/287

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH
https://www.wjgnet.com/bpg/gerinfo/240

PUBLICATION MISCONDUCT
https://www.wjgnet.com/bpg/gerinfo/208

ARTICLE PROCESSING CHARGE
https://www.wjgnet.com/bpg/gerinfo/242

STEPS FOR SUBMITTING MANUSCRIPTS
https://www.wjgnet.com/bpg/GerInfo/239

ONLINE SUBMISSION
https://www.f6publishing.com

World Journal of Stem Cells

Submit a Manuscript: https://www.f6publishing.com

World J Stem Cells 2019 November 26; 11(11): 937-956

DOI: 10.4252/wjsc.v11.i11.937

ISSN 1948-0210 (online)

REVIEW

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

Maria Teresa Valenti, Michela Serena, Luca Dalle Carbonare, Donato Zipeto

ORCID number: Maria Teresa Valenti (0000-0003-1166-8033); Michela Serena (0000-0002-4697-2003); Luca Dalle Carbonare (0000-0003-3263-6671); Donato Zipeto (0000-0002-2168-4144).

Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting, critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Manuscript source: Invited manuscript

Received: March 23, 2019
Peer-review started: March 23, 2019
First decision: August 1, 2019
Revised: August 12, 2019
Accepted: September 11, 2019
Article in press: September 11, 2019
Published online: November 26, 2019

P-Reviewer: Li SC ,Tanabe S

Maria Teresa Valenti, Luca Dalle Carbonare, Department of Medicine, Section of Internal Medicine D, University of Verona, Verona 37134, Italy

Michela Serena, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom

Donato Zipeto, Department of Neurosciences, Biomedicine and Movement Sciences, Laboratory of Molecular Biology, Verona 37134, Italy

Corresponding author: Maria Teresa Valenti, BSc, PhD, Research Fellow, Department of Medicine, University of Verona, Ple Scuro 10, Verona 37100, Italy.
mariateresa.valenti@univr.it
Telephone: +39-45-8128450
Fax: +39-45-8027403

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

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

S-Editor: Zhang L
L-Editor: Filipodia
E-Editor: Zhou BX

degenerative diseases.

Citation: Valenti MT, Serena M, Carbonare LD, Zipeto D. CRISPR/Cas system: An emerging technology in stem cell research. *World J Stem Cells* 2019; 11(11): 937-956
URL: https://www.wjgnet.com/1948-0210/full/v11/i11/937.htm
DOI: https://dx.doi.org/10.4252/wjsc.v11.i11.937

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 (CCR5Δ32/Δ32) 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 Hhigher Eeukaryotes and Pprokaryotes Nnucleotide-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[32] 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 ands 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 *et al*[163], 2018	Spinal muscular atrophy
Calvo-Garrido *et al*[164], 2019	Neuronal
Dong *et al*[165], 2019	Hereditary hearing loss
Zhao *et al*[166], 2019	Breast cancer
Yanagihara *et al*[167], 2019	Skeletal diseases
Vrugt *et al*[168], 2019	Fanconi anemia
Blanas *et al*[169], 2019	Colorectal cancer
Sun *et al*[170], 2019	Glioblastoma
Jelinkova *et al*[171], 2019	Duchenne muscular dystrophy
Hurtado *et al*[172], 2018	Renal
Tang *et al*[173], 2019	Cardiac hypertrophy
Tian *et al*[174], 2019	Pediatric biliary atresia
Wang *et al*[175], 2018	Werner syndrome
Barnes *et al*[176], 2018	Neuronal
Frasier *et al*[177], 2018	Cardiac arrhythmia
Sasaki-Honda *et al*[178], 2018	facioscapulohumeral muscular dystrophy
Wang *et al*[179], 2018	Hepatoma
Moghaddas *et al*[180], 2018	Autoinflammatory
Liu *et al*[181], 2018	Colon cancer
Jiao *et al*[182], 2018	Cardiac disorders
Lyu *et al*[183], 2018	Haemophilia
Deng *et al*[184], 2018	Retinitis pigmentosa
Wattanapanitch *et al*[185], 2018	Thalassemia
Suda *et al*[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 pyrogenes* 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.

REFERENCES

1 **Capecchi MR**. Altering the genome by homologous recombination. *Science* 1989; **244**: 1288-1292 [PMID: 2660260 DOI: 10.1126/science.2660260]

2 **Rouet P**, Smih F, Jasin M. Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Mol Cell Biol* 1994; **14**: 8096-8106 [PMID: 7969147 DOI: 10.1128/mcb.14.12.8096]

3 **Choulika A**, Perrin A, Dujon B, Nicolas JF. Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of Saccharomyces cerevisiae. *Mol Cell Biol* 1995; **15**: 1968-1973 [PMID: 7891691 DOI: 10.1128/mcb.15.4.1968]

4 **Urnov FD**, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Porteus MH, Gregory PD, Holmes MC. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* 2005; **435**: 646-651 [PMID: 15806097 DOI: 10.1038/nature03556]

5 **Christian M**, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, Bogdanove AJ, Voytas DF. Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 2010; **186**: 757-761 [PMID: 20660643 DOI: 10.1534/genetics.110.120717]

6 **Gaj T**, Gersbach CA, Barbas CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* 2013; **31**: 397-405 [PMID: 23664777 DOI: 10.1016/j.tibtech.2013.04.004]

7 **Deng D**, Yan C, Pan X, Mahfouz M, Wang J, Zhu JK, Shi Y, Yan N. Structural basis for sequence-specific recognition of DNA by TAL effectors. *Science* 2012; **335**: 720-723 [PMID: 22223738 DOI: 10.1126/science.1215670]

8 **Li H**, Haurigot V, Doyon Y, Li T, Wong SY, Bhagwat AS, Malani N, Anguela XM, Sharma R, Ivanciu L, Murphy SL, Finn JD, Khazi FR, Zhou S, Paschon DE, Rebar EJ, Bushman FD, Gregory PD, Holmes MC, High KA. In vivo genome editing restores haemostasis in a mouse model of haemophilia. *Nature* 2011; **475**: 217-221 [PMID: 21706032 DOI: 10.1038/nature10177]

9 **Sebastiano V**, Maeder ML, Angstman JF, Haddad B, Khayter C, Yeo DT, Goodwin MJ, Hawkins JS, Ramirez CL, Batista LF, Artandi SE, Wernig M, Joung JK. In situ genetic correction of the sickle cell anemia mutation in human induced pluripotent stem cells using engineered zinc finger nucleases. *Stem Cells* 2011; **29**: 1717-1726 [PMID: 21898685 DOI: 10.1002/stem.718]

10 **Zou J**, Mali P, Huang X, Dowey SN, Cheng L. Site-specific gene correction of a point mutation in human iPS cells derived from an adult patient with sickle cell disease. *Blood* 2011; **118**: 4599-4608 [PMID: 21881051 DOI: 10.1182/blood-2011-02-335554]

11 **Perez EE**, Wang J, Miller JC, Jouvenot Y, Kim KA, Liu O, Wang N, Lee G, Bartsevich VV, Lee YL, Guschin DY, Rupniewski I, Waite AJ, Carpenito C, Carroll RG, Orange JS, Urnov FD, Rebar EJ, Ando D, Gregory PD, Riley JL, Holmes MC, June CH. Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nat Biotechnol* 2008; **26**: 808-816 [PMID: 18587387 DOI: 10.1038/nbt1410]

12 **Holt N**, Wang J, Kim K, Friedman G, Wang X, Taupin V, Crooks GM, Kohn DB, Gregory PD, Holmes MC, Cannon PM. Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted to CCR5 control HIV-1 in vivo. *Nat Biotechnol* 2010; **28**: 839-847 [PMID: 20601939 DOI: 10.1038/nbt.1663]

13 **Voit RA**, McMahon MA, Sawyer SL, Porteus MH. Generation of an HIV resistant T-cell line by targeted "stacking" of restriction factors. *Mol Ther* 2013; **21**: 786-795 [PMID: 23358186 DOI: 10.1038/mt.2012.284]

14 **Hütter G**, Nowak D, Mossner M, Ganepola S, Müssig A, Allers K, Schneider T, Hofmann J, Kücherer C, Blau O, Blau IW, Hofmann WK, Thiel E. Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N Engl J Med* 2009; **360**: 692-698 [PMID: 19213682 DOI: 10.1056/NEJMoa0802905]

15 **Gupta RK**, Abdul-Jawad S, McCoy LE, Mok HP, Peppa D, Salgado M, Martinez-Picado J, Nijhuis M, Wensing AMJ, Lee H, Grant P, Nastouli E, Lambert J, Pace M, Salasc F, Monit C, Innes AJ, Muir L, Waters L, Frater J, Lever AML, Edwards SG, Gabriel IH, Olavarria E. HIV-1 remission following CCR5Δ32/Δ32 haematopoietic stem-cell transplantation. *Nature* 2019; **568**: 244-248 [PMID: 30836379 DOI: 10.1038/s41586-019-1027-4]

16 **Ma N**, Liao B, Zhang H, Wang L, Shan Y, Xue Y, Huang K, Chen S, Zhou X, Chen Y, Pei D, Pan G. Transcription activator-like effector nuclease (TALEN)-mediated gene correction in integration-free β-thalassemia induced pluripotent stem cells. *J Biol Chem* 2013; **288**: 34671-34679 [PMID: 24155235 DOI: 10.1074/jbc.M113.496174]

17 **Li T**, Liu B, Chen CY, Yang B. TALEN-Mediated Homologous Recombination Produces Site-Directed DNA Base Change and Herbicide-Resistant Rice. *J Genet Genomics* 2016; **43**: 297-305 [PMID: 27180265 DOI: 10.1016/j.jgg.2016.03.005]

18 **Qasim W**, Zhan H, Samarasinghe S, Adams S, Amrolia P, Stafford S, Butler K, Rivat C, Wright G,

Somana K, Ghorashian S, Pinner D, Ahsan G, Gilmour K, Lucchini G, Inglott S, Mifsud W, Chiesa R, Peggs KS, Chan L, Farzeneh F, Thrasher AJ, Vora A, Pule M, Veys P. Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. *Sci Transl Med* 2017; 9 [PMID: 28123068 DOI: 10.1126/scitranslmed.aaj2013]

19 **Gasiunas G**, Barrangou R, Horvath P, Siksnys V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc Natl Acad Sci U S A* 2012; **109**: E2579-E2586 [PMID: 22949671 DOI: 10.1073/pnas.1208507109]

20 **Ishino Y**, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *J Bacteriol* 1987; **169**: 5429-5433 [PMID: 3316184 DOI: 10.1128/jb.169.12.5429-5433.1987]

21 **Barrangou R**, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 2007; **315**: 1709-1712 [PMID: 17379808 DOI: 10.1126/science.1138140]

22 **Jansen R**, Embden JD, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. *Mol Microbiol* 2002; **43**: 1565-1575 [PMID: 11952905 DOI: 10.1046/j.1365-2958.2002.02839.x]

23 **Tang TH**, Bachellerie JP, Rozhdestvensky T, Bortolin ML, Huber H, Drungowski M, Elge T, Brosius J, Hüttenhofer A. Identification of 86 candidates for small non-messenger RNAs from the archaeon Archaeoglobus fulgidus. *Proc Natl Acad Sci U S A* 2002; **99**: 7536-7541 [PMID: 12032318 DOI: 10.1073/pnas.112047299]

24 **Mojica FJ**, Díez-Villaseñor C, García-Martínez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol* 2005; **60**: 174-182 [PMID: 15791728 DOI: 10.1007/s00239-004-0046-3]

25 **Rath D**, Amlinger L, Rath A, Lundgren M. The CRISPR-Cas immune system: biology, mechanisms and applications. *Biochimie* 2015; **117**: 119-128 [PMID: 25868999 DOI: 10.1016/j.biochi.2015.03.025]

26 **Deltcheva E**, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 2011; **471**: 602-607 [PMID: 21455174 DOI: 10.1038/nature09886]

27 **Jinek M**, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012; **337**: 816-821 [PMID: 22745249 DOI: 10.1126/science.1225829]

28 **Makarova KS**, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ, Barrangou R, Brouns SJ, Charpentier E, Haft DH, Horvath P, Moineau S, Mojica FJ, Terns RM, Terns MP, White MF, Yakunin AF, Garrett RA, van der Oost J, Backofen R, Koonin EV. An updated evolutionary classification of CRISPR-Cas systems. *Nat Rev Microbiol* 2015; **13**: 722-736 [PMID: 26411297 DOI: 10.1038/nrmicro3569]

29 **Bolotin A**, Quinquis B, Sorokin A, Ehrlich SD. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* 2005; **151**: 2551-2561 [PMID: 16079334 DOI: 10.1099/mic.0.28048-0]

30 **Mojica FJ**, Díez-Villaseñor C, García-Martínez J, Almendros C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* 2009; **155**: 733-740 [PMID: 19246744 DOI: 10.1099/mic.0.023960-0]

31 **Hwang WY**, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* 2013; **31**: 227-229 [PMID: 23360964 DOI: 10.1038/nbt.2501]

32 **Ran FA**, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, Zhang F. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 2013; **154**: 1380-1389 [PMID: 23992846 DOI: 10.1016/j.cell.2013.08.021]

33 **Cho SW**, Kim S, Kim JM, Kim JS. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat Biotechnol* 2013; **31**: 230-232 [PMID: 23360966 DOI: 10.1038/nbt.2507]

34 **Jinek M**, East A, Cheng A, Lin S, Ma E, Doudna J. RNA-programmed genome editing in human cells. *Elife* 2013; **2**: e00471 [PMID: 23386978 DOI: 10.7554/eLife.00471]

35 **Mali P**, Esvelt KM, Church GM. Cas9 as a versatile tool for engineering biology. *Nat Methods* 2013; **10**: 957-963 [PMID: 24076990 DOI: 10.1038/nmeth.2649]

36 **Shen B**, Zhang J, Wu H, Wang J, Ma K, Li Z, Zhang X, Zhang P, Huang X. Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. *Cell Res* 2013; **23**: 720-723 [PMID: 23545779 DOI: 10.1038/cr.2013.46]

37 **Cong L**, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science* 2013; **339**: 819-823 [PMID: 23287718 DOI: 10.1126/science.1231143]

38 **Zetsche B**, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, van der Oost J, Regev A, Koonin EV, Zhang F. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 2015; **163**: 759-771 [PMID: 26422227 DOI: 10.1016/j.cell.2015.09.038]

39 **Shmakov S**, Smargon A, Scott D, Cox D, Pyzocha N, Yan W, Abudayyeh OO, Gootenberg JS, Makarova KS, Wolf YI, Severinov K, Zhang F, Koonin EV. Diversity and evolution of class 2 CRISPR-Cas systems. *Nat Rev Microbiol* 2017; **15**: 169-182 [PMID: 28111461 DOI: 10.1038/nrmicro.2016.184]

40 **Yan WX**, Chong S, Zhang H, Makarova KS, Koonin EV, Cheng DR, Scott DA. Cas13d Is a Compact RNA-Targeting Type VI CRISPR Effector Positively Modulated by a WYL-Domain-Containing Accessory Protein. *Mol Cell* 2018; **70**: 327-339.e5 [PMID: 29551514 DOI: 10.1016/j.molcel.2018.02.028]

41 **Konermann S**, Lotfy P, Brideau NJ, Oki J, Shokhirev MN, Hsu PD. Transcriptome Engineering with RNA-Targeting Type VI-D CRISPR Effectors. *Cell* 2018; **173**: 665-676.e14 [PMID: 29551272 DOI: 10.1016/j.cell.2018.02.033]

42 **East-Seletsky A**, O'Connell MR, Knight SC, Burstein D, Cate JH, Tjian R, Doudna JA. Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. *Nature* 2016; **538**: 270-273 [PMID: 27669025 DOI: 10.1038/nature19802]

43 **Smargon AA**, Cox DBT, Pyzocha NK, Zheng K, Slaymaker IM, Gootenberg JS, Abudayyeh OA, Essletzbichler P, Shmakov S, Makarova KS, Koonin EV, Zhang F. Cas13b Is a Type VI-B CRISPR-Associated RNA-Guided RNase Differentially Regulated by Accessory Proteins Csx27 and Csx28. *Mol Cell* 2017; **65**: 618-630.e7 [PMID: 28065598 DOI: 10.1016/j.molcel.2016.12.023]

44 **O'Connell MR**. Molecular Mechanisms of RNA Targeting by Cas13-containing Type VI CRISPR-Cas Systems. *J Mol Biol* 2019; **431**: 66-87 [PMID: 29940185 DOI: 10.1016/j.jmb.2018.06.029]

45 **Abudayyeh OO**, Gootenberg JS, Essletzbichler P, Han S, Joung J, Belanto JJ, Verdine V, Cox DBT,

Kellner MJ, Regev A, Lander ES, Voytas DF, Ting AY, Zhang F. RNA targeting with CRISPR-Cas13. *Nature* 2017; **550**: 280-284 [PMID: 28976959 DOI: 10.1038/nature24049]

46 **Cox DBT**, Gootenberg JS, Abudayyeh OO, Franklin B, Kellner MJ, Joung J, Zhang F. RNA editing with CRISPR-Cas13. *Science* 2017; **358**: 1019-1027 [PMID: 29070703 DOI: 10.1126/science.aaq0180]

47 **Thomson JA**, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998; **282**: 1145-1147 [PMID: 9804556 DOI: 10.1126/science.282.5391.1145]

48 **Takahashi K**, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861-872 [PMID: 18035408 DOI: 10.1016/j.cell.2007.11.019]

49 **Park IH**, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 2008; **451**: 141-146 [PMID: 18157115 DOI: 10.1038/nature06534]

50 **Tachibana M**, Amato P, Sparman M, Gutierrez NM, Tippner-Hedges R, Ma H, Kang E, Fulati A, Lee HS, Sritanaudomchai H, Masterson K, Larson J, Eaton D, Sadler-Fredd K, Battaglia D, Lee D, Wu D, Jensen J, Patton P, Gokhale S, Stouffer RL, Wolf D, Mitalipov S. Human embryonic stem cells derived by somatic cell nuclear transfer. *Cell* 2013; **153**: 1228-1238 [PMID: 23683578 DOI: 10.1016/j.cell.2013.05.006]

51 **Lian X**, Zhang J, Azarin SM, Zhu K, Hazeltine LB, Bao X, Hsiao C, Kamp TJ, Palecek SP. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. *Nat Protoc* 2013; **8**: 162-175 [PMID: 23257984 DOI: 10.1038/nprot.2012.150]

52 **Takahashi K**, Okita K, Nakagawa M, Yamanaka S. Induction of pluripotent stem cells from fibroblast cultures. *Nat Protoc* 2007; **2**: 3081-3089 [PMID: 18079707 DOI: 10.1038/nprot.2007.418]

53 **Chen SM**, Lee MS, Chang CY, Lin SZ, Cheng EH, Liu YH, Pan HC, Lee HC, Su HL. Prerequisite OCT4 Maintenance Potentiates the Neural Induction of Differentiating Human Embryonic Stem Cells and Induced Pluripotent Stem Cells. *Cell Transplant* 2015; **24**: 829-844 [PMID: 24256943 DOI: 10.3727/096368913X675179]

54 **Krohne TU**, Westenskow PD, Kurihara T, Friedlander DF, Lehmann M, Dorsey AL, Li W, Zhu S, Schultz A, Wang J, Siuzdak G, Ding S, Friedlander M. Generation of retinal pigment epithelial cells from small molecules and OCT4 reprogrammed human induced pluripotent stem cells. *Stem Cells Transl Med* 2012; **1**: 96-109 [PMID: 22532929 DOI: 10.5966/sctm.2011-0057]

55 **Yu J**, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007; **318**: 1917-1920 [PMID: 18029452 DOI: 10.1126/science.1151526]

56 **Buganim Y**, Jaenisch R. Transdifferentiation by defined factors as a powerful research tool to address basic biological questions. *Cell Cycle* 2012; **11**: 4485-4486 [PMID: 23165203 DOI: 10.4161/cc.22665]

57 **Moon JH**, Heo JS, Kwon S, Kim J, Hwang J, Kang PJ, Kim A, Kim HO, Whang KY, Yoon BS, You S. Two-step generation of induced pluripotent stem cells from mouse fibroblasts using Id3 and Oct4. *J Mol Cell Biol* 2012; **4**: 59-62 [PMID: 22131360 DOI: 10.1093/jmcb/mjr038]

58 **Judson RL**, Babiarz JE, Venere M, Blelloch R. Embryonic stem cell-specific microRNAs promote induced pluripotency. *Nat Biotechnol* 2009; **27**: 459-461 [PMID: 19363475 DOI: 10.1038/nbt.1535]

59 **Anokye-Danso F**, Trivedi CM, Juhr D, Gupta M, Cui Z, Tian Y, Zhang Y, Yang W, Gruber PJ, Epstein JA, Morrisey EE. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell Stem Cell* 2011; **8**: 376-388 [PMID: 21474102 DOI: 10.1016/j.stem.2011.03.001]

60 **Yang J**, Li S, He XB, Cheng C, Le W. Induced pluripotent stem cells in Alzheimer's disease: applications for disease modeling and cell-replacement therapy. *Mol Neurodegener* 2016; **11**: 39 [PMID: 27184028 DOI: 10.1186/s13024-016-0106-3]

61 **DeWitt MA**, Magis W, Bray NL, Wang T, Berman JR, Urbinati F, Heo SJ, Mitros T, Muñoz DP, Boffelli D, Kohn DB, Walters MC, Carroll D, Martin DI, Corn JE. Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells. *Sci Transl Med* 2016; **8**: 360ra134 [PMID: 27733558 DOI: 10.1126/scitranslmed.aaf9336]

62 **Dalle Carbonare L**, Matte A, Valenti MT, Siciliano A, Cristellon A, De Franceschi L. Zoledronic Acid Reverses Acute Bone Impairment in a Mouse Model for Sickle Cell Disease. *Blood* 2014; **124**: 122

63 **Lucas D**, O'Leary HA, Ebert BL, Cowan CA, Tremblay CS. Utility of CRISPR/Cas9 systems in hematology research. *Exp Hematol* 2017; **54**: 1-3 [PMID: 28668351 DOI: 10.1016/j.exphem.2017.06.006]

64 **Heckl D**, Kowalczyk MS, Yudovich D, Belizaire R, Puram RV, McConkey ME, Thielke A, Aster JC, Regev A, Ebert BL. Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing. *Nat Biotechnol* 2014; **32**: 941-946 [PMID: 24952903 DOI: 10.1038/nbt.2951]

65 **Bejar R**, Lord A, Stevenson K, Bar-Natan M, Pérez-Ladaga A, Zaneveld J, Wang H, Caughey B, Stojanov P, Getz G, Garcia-Manero G, Kantarjian H, Chen R, Stone RM, Neuberg D, Steensma DP, Ebert BL. TET2 mutations predict response to hypomethylating agents in myelodysplastic syndrome patients. *Blood* 2014; **124**: 2705-2712 [PMID: 25224413 DOI: 10.1182/blood-2014-06-582809]

66 **Donahue DA**, Wainberg MA. Cellular and molecular mechanisms involved in the establishment of HIV-1 latency. *Retrovirology* 2013; **10**: 11 [PMID: 23375003 DOI: 10.1186/1742-4690-10-11]

67 **Zhu W**, Lei R, Le Duff Y, Li J, Guo F, Wainberg MA, Liang C. The CRISPR/Cas9 system inactivates latent HIV-1 proviral DNA. *Retrovirology* 2015; **12**: 22 [PMID: 25808449 DOI: 10.1186/s12977-015-0150-z]

68 **Ebina H**, Misawa N, Kanemura Y, Koyanagi Y. Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. *Sci Rep* 2013; **3**: 2510 [PMID: 23974631 DOI: 10.1038/srep02510]

69 **Kaminski R**, Bella R, Yin C, Otte J, Ferrante P, Gendelman HE, Li H, Booze R, Gordon J, Hu W, Khalili K. Excision of HIV-1 DNA by gene editing: a proof-of-concept in vivo study. *Gene Ther* 2016; **23**: 696 [PMID: 27488023 DOI: 10.1038/gt.2016.45]

70 **Hu W**, Kaminski R, Yang F, Zhang Y, Cosentino L, Li F, Luo B, Alvarez-Carbonell D, Garcia-Mesa Y, Karn J, Mo X, Khalili K. RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. *Proc Natl Acad Sci U S A* 2014; **111**: 11461-11466 [PMID: 25049410 DOI: 10.1073/pnas.1405186111]

71 **Yin C**, Zhang T, Qu X, Zhang Y, Putatunda R, Xiao X, Li F, Xiao W, Zhao H, Dai S, Qin X, Mo X, Young WB, Khalili K, Hu W. In Vivo Excision of HIV-1 Provirus by saCas9 and Multiplex Single-Guide RNAs in Animal Models. *Mol Ther* 2017; **25**: 1168-1186 [PMID: 28366764 DOI: 10.1016/j.ymthe.2017.03.012]

72 **Konermann S**, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H, Nureki O, Zhang F. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* 2015; **517**: 583-588 [PMID: 25494202 DOI: 10.1038/nature14136]

73 **Zhang Y**, Yin C, Zhang T, Li F, Yang W, Kaminski R, Fagan PR, Putatunda R, Young WB, Khalili K, Hu W. CRISPR/gRNA-directed synergistic activation mediator (SAM) induces specific, persistent and robust reactivation of the HIV-1 latent reservoirs. *Sci Rep* 2015; **5**: 16277 [PMID: 26538064 DOI: 10.1038/srep16277]

74 **Nakamoto FK**, Okamoto S, Mitsui J, Sone T, Ishikawa M, Yamamoto Y, Kanegae Y, Nakatake Y, Imaizumi K, Ishiura H, Tsuji S, Okano H. The pathogenesis linked to coenzyme Q10 insufficiency in iPSC-derived neurons from patients with multiple-system atrophy. *Sci Rep* 2018; **8**: 14215 [PMID: 30242188 DOI: 10.1038/s41598-018-32573-1]

75 **Bettens K**, Sleegers K, Van Broeckhoven C. Genetic insights in Alzheimer's disease. *Lancet Neurol* 2013; **12**: 92-104 [PMID: 23237904 DOI: 10.1016/S1474-4422(12)70259-4]

76 **Schellenberg GD**, Bird TD, Wijsman EM, Orr HT, Anderson L, Nemens E, White JA, Bonnycastle L, Weber JL, Alonso ME. Genetic linkage evidence for a familial Alzheimer's disease locus on chromosome 14. *Science* 1992; **258**: 668-671 [PMID: 1411576 DOI: 10.1126/science.1411576]

77 **Ortiz-Virumbrales M**, Moreno CL, Kruglikov I, Marazuela P, Sproul A, Jacob S, Zimmer M, Paull D, Zhang B, Schadt EE, Ehrlich ME, Tanzi RE, Arancio O, Noggle S, Gandy S. CRISPR/Cas9-Correctable mutation-related molecular and physiological phenotypes in iPSC-derived Alzheimer's PSEN2 N141I neurons. *Acta Neuropathol Commun* 2017; **5**: 77 [PMID: 29078805 DOI: 10.1186/s40478-017-0475-z]

78 **Eisenstein M**. Genetics: finding risk factors. *Nature* 2011; **475**: S20-S22 [PMID: 21760580 DOI: 10.1038/475S20a]

79 **Rohn TT**, Kim N, Isho NF, Mack JM. The Potential of CRISPR/Cas9 Gene Editing as a Treatment Strategy for Alzheimer's Disease. *J Alzheimers Dis Parkinsonism* 2018; 8 [PMID: 30090689 DOI: 10.4172/2161-0460.1000439]

80 **Golas MM**, Sander B. Use of human stem cells in Huntington disease modeling and translational research. *Exp Neurol* 2016; **278**: 76-90 [PMID: 26826449 DOI: 10.1016/j.expneurol.2016.01.021]

81 **Rosati J**, Bidollari E, Rotundo G, Ferrari D, Torres B, Bernardini L, Consoli F, De Luca A, Santimone I, Lamorte G, Squitieri F, Vescovi AL. Generation of induced pluripotent stem cell line, CSSi002-A (2851), from a patient with juvenile Huntington Disease. *Stem Cell Res* 2018; **27**: 86-89 [PMID: 29342448 DOI: 10.1016/j.scr.2018.01.011]

82 **Golas MM**. Human cellular models of medium spiny neuron development and Huntington disease. *Life Sci* 2018; **209**: 179-196 [PMID: 30031060 DOI: 10.1016/j.lfs.2018.07.030]

83 **Saito A**, Ooki A, Nakamura T, Onodera S, Hayashi K, Hasegawa D, Okudaira T, Watanabe K, Kato H, Onda T, Watanabe A, Kosaki K, Nishimura K, Ohtaka M, Nakanishi M, Sakamoto T, Yamaguchi A, Sueishi K, Azuma T. Targeted reversion of induced pluripotent stem cells from patients with human cleidocranial dysplasia improves bone regeneration in a rat calvarial bone defect model. *Stem Cell Res Ther* 2018; **9**: 12 [PMID: 29357927 DOI: 10.1186/s13287-017-0754-4]

84 **Ducy P**, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Karsenty G. Increased bone formation in osteocalcin-deficient mice. *Nature* 1996; **382**: 448-452 [PMID: 8684484 DOI: 10.1038/382448a0]

85 **Lambert LJ**, Challa AK, Niu A, Zhou L, Tucholski J, Johnson MS, Nagy TR, Eberhardt AW, Estep PN, Kesterson RA, Grams JM. Increased trabecular bone and improved biomechanics in an osteocalcin-null rat model created by CRISPR/Cas9 technology. *Dis Model Mech* 2016; **9**: 1169-1179 [PMID: 27483347 DOI: 10.1242/dmm.025247]

86 **Long C**, McAnally JR, Shelton JM, Mireault AA, Bassel-Duby R, Olson EN. Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science* 2014; **345**: 1184-1188 [PMID: 25123483 DOI: 10.1126/science.1254445]

87 **Lapidos KA**, Kakkar R, McNally EM. The dystrophin glycoprotein complex: signaling strength and integrity for the sarcolemma. *Circ Res* 2004; **94**: 1023-1031 [PMID: 15117830 DOI: 10.1161/01.RES.0000126574.61061.25]

88 **Li HL**, Fujimoto N, Sasakawa N, Shirai S, Ohkame T, Sakuma T, Tanaka M, Amano N, Watanabe A, Sakurai H, Yamamoto T, Yamanaka S, Hotta A. Precise correction of the dystrophin gene in duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9. *Stem Cell Reports* 2015; **4**: 143-154 [PMID: 25434822 DOI: 10.1016/j.stemcr.2014.10.013]

89 **van Gelder CM**, Vollebregt AA, Plug I, van der Ploeg AT, Reuser AJ. Treatment options for lysosomal storage disorders: developing insights. *Expert Opin Pharmacother* 2012; **13**: 2281-2299 [PMID: 23009070 DOI: 10.1517/14656566.2012.729039]

90 **van der Ploeg AT**, Reuser AJ. Pompe's disease. *Lancet* 2008; **372**: 1342-1353 [PMID: 18929906 DOI: 10.1016/S0140-6736(08)61555-X]

91 **van Til NP**, Stok M, Aerts Kaya FS, de Waard MC, Farahbakhshian E, Visser TP, Kroos MA, Jacobs EH, Willart MA, van der Wegen P, Scholte BJ, Lambrecht BN, Duncker DJ, van der Ploeg AT, Reuser AJ, Verstegen MM, Wagemaker G. Lentiviral gene therapy of murine hematopoietic stem cells ameliorates the Pompe disease phenotype. *Blood* 2010; **115**: 5329-5337 [PMID: 20385789 DOI: 10.1182/blood-2009-11-252874]

92 **The European Heart Network**. European Cardiovascular Disease Statistics 2017. Available from: http://www.ehnheart.org/cvd-statistics.html

93 **Louch WE**, Sheehan KA, Wolska BM. Methods in cardiomyocyte isolation, culture, and gene transfer. *J Mol Cell Cardiol* 2011; **51**: 288-298 [PMID: 21723873 DOI: 10.1016/j.yjmcc.2011.06.012]

94 **Niu Y**, Shen B, Cui Y, Chen Y, Wang J, Wang L, Kang Y, Zhao X, Si W, Li W, Xiang AP, Zhou J, Guo X, Bi Y, Si C, Hu B, Dong G, Wang H, Zhou Z, Li T, Tan T, Pu X, Wang F, Ji S, Zhou Q, Huang X, Ji W, Sha J. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell* 2014; **156**: 836-843 [PMID: 24486104 DOI: 10.1016/j.cell.2014.01.027]

95 **Shao Y**, Guan Y, Wang L, Qiu Z, Liu M, Chen Y, Wu L, Li Y, Ma X, Liu M, Li D. CRISPR/Cas-mediated genome editing in the rat via direct injection of one-cell embryos. *Nat Protoc* 2014; **9**: 2493-2512 [PMID: 25255092 DOI: 10.1038/nprot.2014.171]

96 **Wang G**, McCain ML, Yang L, He A, Pasqualini FS, Agarwal A, Yuan H, Jiang D, Zhang D, Zangi L, Geva J, Roberts AE, Ma Q, Ding J, Chen J, Wang DZ, Li K, Wang J, Wanders RJ, Kulik W, Vaz FM, Laflamme MA, Murry CE, Chien KR, Kelley RI, Church GM, Parker KK, Pu WT. Modeling the mitochondrial cardiomyopathy of Barth syndrome with induced pluripotent stem cell and heart-on-chip

technologies. *Nat Med* 2014; **20**: 616-623 [PMID: 24813252 DOI: 10.1038/nm.3545]
97 **Hinson JT**, Chopra A, Nafissi N, Polacheck WJ, Benson CC, Swist S, Gorham J, Yang L, Schafer S, Sheng CC, Haghighi A, Homsy J, Hubner N, Church G, Cook SA, Linke WA, Chen CS, Seidman JG, Seidman CE. HEART DISEASE. Titin mutations in iPS cells define sarcomere insufficiency as a cause of dilated cardiomyopathy. *Science* 2015; **349**: 982-986 [PMID: 26315439 DOI: 10.1126/science.aaa5458]
98 **Denjoy I**, Lupoglazoff JM, Villain E, Vaksmann G, Godart F, Lucet V, Leenhardt A, Guicheney P, Schwartz P. [The Jervell and Lange-Nielsen syndrome. Natural history, molecular basis and clinical outcome]. *Arch Mal Coeur Vaiss* 2007; **100**: 359-364 [PMID: 17646758]
99 **Yamamoto Y**, Makiyama T, Harita T, Sasaki K, Wuriyanghai Y, Hayano M, Nishiuchi S, Kohjitani H, Hirose S, Chen J, Yokoi F, Ishikawa T, Ohno S, Chonabayashi K, Motomura H, Yoshida Y, Horie M, Makita N, Kimura T. Allele-specific ablation rescues electrophysiological abnormalities in a human iPS cell model of long-QT syndrome with a CALM2 mutation. *Hum Mol Genet* 2017; **26**: 1670-1677 [PMID: 28335032 DOI: 10.1093/hmg/ddx073]
100 **Beaudoin M**, Gupta RM, Won HH, Lo KS, Do R, Henderson CA, Lavoie-St-Amour C, Langlois S, Rivas D, Lehoux S, Kathiresan S, Tardif JC, Musunuru K, Lettre G. Myocardial Infarction-Associated SNP at 6p24 Interferes With MEF2 Binding and Associates With PHACTR1 Expression Levels in Human Coronary Arteries. *Arterioscler Thromb Vasc Biol* 2015; **35**: 1472-1479 [PMID: 25838425 DOI: 10.1161/ATVBAHA.115.305534]
101 **Mosqueira D**, Mannhardt I, Bhagwan JR, Lis-Slimak K, Katili P, Scott E, Hassan M, Prondzynski M, Harmer SC, Tinker A, Smith JGW, Carrier L, Williams PM, Gaffney D, Eschenhagen T, Hansen A, Denning C. CRISPR/Cas9 editing in human pluripotent stem cell-cardiomyocytes highlights arrhythmias, hypocontractility, and energy depletion as potential therapeutic targets for hypertrophic cardiomyopathy. *Eur Heart J* 2018; **39**: 3879-3892 [PMID: 29741611 DOI: 10.1093/eurheartj/ehy249]
102 **Balboa D**, Saarimäki-Vire J, Otonkoski T. Concise Review: Human Pluripotent Stem Cells for the Modeling of Pancreatic β-Cell Pathology. *Stem Cells* 2019; **37**: 33-41 [PMID: 30270471 DOI: 10.1002/stem.2913]
103 **Coombe L**, Kadri A, Martinez JF, Tatachar V, Gallicano GI. Current approaches in regenerative medicine for the treatment of diabetes: introducing CRISPR/CAS9 technology and the case for non-embryonic stem cell therapy. *Am J Stem Cells* 2018; **7**: 104-113 [PMID: 30697454]
104 **Beer NL**, Gloyn AL. Genome-edited human stem cell-derived beta cells: a powerful tool for drilling down on type 2 diabetes GWAS biology. *F1000Res* 2016; 5 [PMID: 27508066 DOI: 10.12688/f1000research.8682.1]
105 **Kyono Y**, Kitzman JO, Parker SCJ. Genomic annotation of disease-associated variants reveals shared functional contexts. *Diabetologia* 2019; **62**: 735-743 [PMID: 30756131 DOI: 10.1007/s00125-019-4823-3]
106 **Balboa D**, Saarimäki-Vire J, Borshagovski D, Survila M, Lindholm P, Galli E, Eurola S, Ustinov J, Grym H, Huopio H, Partanen J, Wartiovaara K, Otonkoski T. Insulin mutations impair beta-cell development in a patient-derived iPSC model of neonatal diabetes. *Elife* 2018; 7 [PMID: 30412052 DOI: 10.7554/eLife.38519]
107 **Lathia JD**, Mack SC, Mulkearns-Hubert EE, Valentim CL, Rich JN. Cancer stem cells in glioblastoma. *Genes Dev* 2015; **29**: 1203-1217 [PMID: 26109046 DOI: 10.1101/gad.261982.115]
108 **Toledo CM**, Ding Y, Hoellerbauer P, Davis RJ, Basom R, Girard EJ, Lee E, Corrin P, Hart T, Bolouri H, Davison J, Zhang Q, Hardcastle J, Aronow BJ, Plaisier CL, Baliga NS, Moffat J, Lin Q, Li XN, Nam DH, Lee J, Pollard SM, Zhu J, Delrow JJ, Clurman BE, Olson JM, Paddison PJ. Genome-wide CRISPR-Cas9 Screens Reveal Loss of Redundancy between PKMYT1 and WEE1 in Glioblastoma Stem-like Cells. *Cell Rep* 2015; **13**: 2425-2439 [PMID: 26673326 DOI: 10.1016/j.celrep.2015.11.021]
109 **Minagawa A**, Yoshikawa T, Yasukawa M, Hotta A, Kunitomo M, Iriguchi S, Takiguchi M, Kassai Y, Imai E, Yasui Y, Kawai Y, Zhang R, Uemura Y, Miyoshi H, Nakanishi M, Watanabe A, Hayashi A, Kawana K, Fujii T, Nakatsura T, Kaneko S. Enhancing T Cell Receptor Stability in Rejuvenated iPSC-Derived T Cells Improves Their Use in Cancer Immunotherapy. *Cell Stem Cell* 2018; **23**: 850-858.e4 [PMID: 30449714 DOI: 10.1016/j.stem.2018.10.005]
110 **Chang CJ**, Kotini AG, Olszewska M, Georgomanoli M, Teruya-Feldstein J, Sperber H, Sanchez R, DeVita R, Martins TJ, Abdel-Wahab O, Bradley RK, Papapetrou EP. Dissecting the Contributions of Cooperating Gene Mutations to Cancer Phenotypes and Drug Responses with Patient-Derived iPSCs. *Stem Cell Reports* 2018; **10**: 1610-1624 [PMID: 29681544 DOI: 10.1016/j.stemcr.2018.03.020]
111 **Wells SA**, Pacini F, Robinson BG, Santoro M. Multiple endocrine neoplasia type 2 and familial medullary thyroid carcinoma: an update. *J Clin Endocrinol Metab* 2013; **98**: 3149-3164 [PMID: 23744408 DOI: 10.1210/jc.2013-1204]
112 **Hadoux J**, Desterke C, Féraud O, Guibert M, De Rose RF, Opolon P, Divers D, Gobbo E, Griscelli F, Schlumberger M, Bennaceur-Griscelli A, Turhan AG. Transcriptional landscape of a RETC634Y-mutated iPSC and its CRISPR-corrected isogenic control reveals the putative role of EGR1 transcriptional program in the development of multiple endocrine neoplasia type 2A-associated cancers. *Stem Cell Res* 2018; **26**: 8-16 [PMID: 29197744 DOI: 10.1016/j.scr.2017.11.015]
113 **O'Geen H**, Yu AS, Segal DJ. How specific is CRISPR/Cas9 really? *Curr Opin Chem Biol* 2015; **29**: 72-78 [PMID: 26517564 DOI: 10.1016/j.cbpa.2015.10.001]
114 **Tan EP**, Li Y, Velasco-Herrera Mdel C, Yusa K, Bradley A. Off-target assessment of CRISPR-Cas9 guiding RNAs in human iPS and mouse ES cells. *Genesis* 2015; **53**: 225-236 [PMID: 25378133 DOI: 10.1002/dvg.22835]
115 **Veres A**, Gosis BS, Ding Q, Collins R, Ragavendran A, Brand H, Erdin S, Cowan CA, Talkowski ME, Musunuru K. Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing. *Cell Stem Cell* 2014; **15**: 27-30 [PMID: 24996167 DOI: 10.1016/j.stem.2014.04.020]
116 **Yang L**, Grishin D, Wang G, Aach J, Zhang CZ, Chari R, Homsy J, Cai X, Zhao Y, Fan JB, Seidman C, Seidman J, Pu W, Church G. Targeted and genome-wide sequencing reveal single nucleotide variations impacting specificity of Cas9 in human stem cells. *Nat Commun* 2014; **5**: 5507 [PMID: 25425480 DOI: 10.1038/ncomms6507]
117 **Schaefer KA**, Wu WH, Colgan DF, Tsang SH, Bassuk AG, Mahajan VB. Unexpected mutations after CRISPR-Cas9 editing in vivo. *Nat Methods* 2017; **14**: 547-548 [PMID: 28557981 DOI: 10.1038/nmeth.4293]
118 CRISPR off-targets: a reassessment. *Nat Methods* 2018; **15**: 229-230 [DOI: 10.1038/Nmeth.4664]
119 **Iyer V**, Boroviak K, Thomas M, Doe B, Riva L, Ryder E, Adams DJ. No unexpected CRISPR-Cas9 off-

target activity revealed by trio sequencing of gene-edited mice. *PLoS Genet* 2018; **14**: e1007503 [PMID: 29985941 DOI: 10.1371/journal.pgen.1007503]
120 **Anderson KR**, Haeussler M, Watanabe C, Janakiraman V, Lund J, Modrusan Z, Stinson J, Bei Q, Buechler A, Yu C, Thamminana SR, Tam L, Sowick MA, Alcantar T, O'Neil N, Li J, Ta L, Lima L, Roose-Girma M, Rairdan X, Durinck S, Warming S. CRISPR off-target analysis in genetically engineered rats and mice. *Nat Methods* 2018; **15**: 512-514 [PMID: 29786090 DOI: 10.1038/s41592-018-0011-5]
121 **Akcakaya P**, Bobbin ML, Guo JA, Malagon-Lopez J, Clement K, Garcia SP, Fellows MD, Porritt MJ, Firth MA, Carreras A, Baccega T, Seeliger F, Bjursell M, Tsai SQ, Nguyen NT, Nitsch R, Mayr LM, Pinello L, Bohlooly-Y M, Aryee MJ, Maresca M, Joung JK. In vivo CRISPR editing with no detectable genome-wide off-target mutations. *Nature* 2018; **561**: 416-419 [PMID: 30209390 DOI: 10.1038/s41586-018-0500-9]
122 **Kim D**, Kim JS. DIG-seq: a genome-wide CRISPR off-target profiling method using chromatin DNA. *Genome Res* 2018; **28**: 1894-1900 [PMID: 30413470 DOI: 10.1101/gr.236620.118]
123 **Kim D**, Bae S, Park J, Kim E, Kim S, Yu HR, Hwang J, Kim JI, Kim JS. Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nat Methods* 2015; **12**: 237-243, 1 p following 243 [PMID: 25664545 DOI: 10.1038/nmeth.3284]
124 **Tsai SQ**, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, Wyvekens N, Khayter C, Iafrate AJ, Le LP, Aryee MJ, Joung JK. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol* 2015; **33**: 187-197 [PMID: 25513782 DOI: 10.1038/nbt.3117]
125 **Frock RL**, Hu J, Meyers RM, Ho YJ, Kii E, Alt FW. Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. *Nat Biotechnol* 2015; **33**: 179-186 [PMID: 25503383 DOI: 10.1038/nbt.3101]
126 **Ravindran S**. New Methods to Detect CRISPR Off-Target Mutations. Available from: https://www.the-scientist.com/lab-tools/new-methods-to-detect-crispr-off-target-mutations-30013
127 **Fu Y**, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* 2013; **31**: 822-826 [PMID: 23792628 DOI: 10.1038/nbt.2623]
128 **Cho SW**, Kim S, Kim Y, Kweon J, Kim HS, Bae S, Kim JS. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res* 2014; **24**: 132-141 [PMID: 24253446 DOI: 10.1101/gr.162339.113]
129 **Casini A**, Olivieri M, Petris G, Montagna C, Reginato G, Maule G, Lorenzin F, Prandi D, Romanel A, Demichelis F, Inga A, Cereseto A. A highly specific SpCas9 variant is identified by in vivo screening in yeast. *Nat Biotechnol* 2018; **36**: 265-271 [PMID: 29431739 DOI: 10.1038/nbt.4066]
130 **Kleinstiver BP**, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JK. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* 2016; **529**: 490-495 [PMID: 26735016 DOI: 10.1038/nature16526]
131 **Vakulskas C**, Collingwood M, Behlke M. High Fidelity Genome Editing with a Novel Mutant HIF1 Cas9. *Mol Ther* 2018; **26**: 92-92
132 **Hou Z**, Zhang Y, Propson NE, Howden SE, Chu LF, Sontheimer EJ, Thomson JA. Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis. *Proc Natl Acad Sci U S A* 2013; **110**: 15644-15649 [PMID: 23940360 DOI: 10.1073/pnas.1313587110]
133 **Kleinstiver BP**, Tsai SQ, Prew MS, Nguyen NT, Welch MM, Lopez JM, McCaw ZR, Aryee MJ, Joung JK. Genome-wide specificities of CRISPR-Cas Cpf1 nucleases in human cells. *Nat Biotechnol* 2016; **34**: 869-874 [PMID: 27347757 DOI: 10.1038/nbt.3620]
134 **Farboud B**, Jarvis E, Roth TL, Shin J, Corn JE, Marson A, Meyer BJ, Patel NH, Hochstrasser ML. Enhanced Genome Editing with Cas9 Ribonucleoprotein in Diverse Cells and Organisms. *J Vis Exp* 2018 [PMID: 29889198 DOI: 10.3791/57350]
135 **Yang H**, Wang H, Shivalila CS, Cheng AW, Shi L, Jaenisch R. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* 2013; **154**: 1370-1379 [PMID: 23992847 DOI: 10.1016/j.cell.2013.08.022]
136 **Genovese P**, Schiroli G, Escobar G, Tomaso TD, Firrito C, Calabria A, Moi D, Mazzieri R, Bonini C, Holmes MC, Gregory PD, van der Burg M, Gentner B, Montini E, Lombardo A, Naldini L. Targeted genome editing in human repopulating haematopoietic stem cells. *Nature* 2014; **510**: 235-240 [PMID: 24870228 DOI: 10.1038/nature13420]
137 **Cox DB**, Platt RJ, Zhang F. Therapeutic genome editing: prospects and challenges. *Nat Med* 2015; **21**: 121-131 [PMID: 25654603 DOI: 10.1038/nm.3793]
138 **Lino CA**, Harper JC, Carney JP, Timlin JA. Delivering CRISPR: a review of the challenges and approaches. *Drug Deliv* 2018; **25**: 1234-1257 [PMID: 29801422 DOI: 10.1080/10717544.2018.1474964]
139 **Wirth T**, Parker N, Ylä-Herttuala S. History of gene therapy. *Gene* 2013; **525**: 162-169 [PMID: 23618815 DOI: 10.1016/j.gene.2013.03.137]
140 **Ran FA**, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F. In vivo genome editing using Staphylococcus aureus Cas9. *Nature* 2015; **520**: 186-191 [PMID: 25830891 DOI: 10.1038/nature14299]
141 **Li L**, He ZY, Wei XW, Gao GP, Wei YQ. Challenges in CRISPR/CAS9 Delivery: Potential Roles of Nonviral Vectors. *Hum Gene Ther* 2015; **26**: 452-462 [PMID: 26176432 DOI: 10.1089/hum.2015.069]
142 **Crudele JM**, Chamberlain JS. Cas9 immunity creates challenges for CRISPR gene editing therapies. *Nat Commun* 2018; **9**: 3497 [PMID: 30158648 DOI: 10.1038/s41467-018-05843-9]
143 **Charlesworth CT**, Deshpande PS, Dever DP, Camarena J, Lemgart VT, Cromer MK, Vakulskas CA, Collingwood MA, Zhang L, Bode NM, Behlke MA, Dejene B, Cieniewicz B, Romano R, Lesch BJ, Gomez-Ospina N, Mantri S, Pavel-Dinu M, Weinberg KI, Porteus MH. Identification of preexisting adaptive immunity to Cas9 proteins in humans. *Nat Med* 2019; **25**: 249-254 [PMID: 30692695 DOI: 10.1038/s41591-018-0326-x]
144 **Wang G**, Zhao N, Berkhout B, Das AT. CRISPR-Cas9 Can Inhibit HIV-1 Replication but NHEJ Repair Facilitates Virus Escape. *Mol Ther* 2016; **24**: 522-526 [PMID: 26796669 DOI: 10.1038/mt.2016.24]
145 **Wang Z**, Pan Q, Gendron P, Zhu W, Guo F, Cen S, Wainberg MA, Liang C. CRISPR/Cas9-Derived Mutations Both Inhibit HIV-1 Replication and Accelerate Viral Escape. *Cell Rep* 2016; **15**: 481-489 [PMID: 27068471 DOI: 10.1016/j.celrep.2016.03.042]
146 **Liao HK**, Gu Y, Diaz A, Marlett J, Takahashi Y, Li M, Suzuki K, Xu R, Hishida T, Chang CJ, Esteban CR, Young J, Izpisua Belmonte JC. Use of the CRISPR/Cas9 system as an intracellular defense against HIV-1 infection in human cells. *Nat Commun* 2015; **6**: 6413 [PMID: 25752527 DOI: 10.1038/ncomms7413]

147 **Vartak SV**, Raghavan SC. Inhibition of nonhomologous end joining to increase the specificity of CRISPR/Cas9 genome editing. *FEBS J* 2015; **282**: 4289-4294 [PMID: 26290158 DOI: 10.1111/febs.13416]

148 **Hou P**, Chen S, Wang S, Yu X, Chen Y, Jiang M, Zhuang K, Ho W, Hou W, Huang J, Guo D. Genome editing of CXCR4 by CRISPR/cas9 confers cells resistant to HIV-1 infection. *Sci Rep* 2015; **5**: 15577 [PMID: 26481100 DOI: 10.1038/srep15577]

149 **Wang W**, Ye C, Liu J, Zhang D, Kimata JT, Zhou P. CCR5 gene disruption via lentiviral vectors expressing Cas9 and single guided RNA renders cells resistant to HIV-1 infection. *PLoS One* 2014; **9**: e115987 [PMID: 25541967 DOI: 10.1371/journal.pone.0115987]

150 **Ye L**, Wang J, Beyer AI, Teque F, Cradick TJ, Qi Z, Chang JC, Bao G, Muench MO, Yu J, Levy JA, Kan YW. Seamless modification of wild-type induced pluripotent stem cells to the natural CCR5Δ32 mutation confers resistance to HIV infection. *Proc Natl Acad Sci U S A* 2014; **111**: 9591-9596 [PMID: 24927590 DOI: 10.1073/pnas.1407473111]

151 **Hultquist JF**, Schumann K, Woo JM, Manganaro L, McGregor MJ, Doudna J, Simon V, Krogan NJ, Marson A. A Cas9 Ribonucleoprotein Platform for Functional Genetic Studies of HIV-Host Interactions in Primary Human T Cells. *Cell Rep* 2016; **17**: 1438-1452 [PMID: 27783955 DOI: 10.1016/j.celrep.2016.09.080]

152 **Liang P**, Xu Y, Zhang X, Ding C, Huang R, Zhang Z, Lv J, Xie X, Chen Y, Li Y, Sun Y, Bai Y, Songyang Z, Ma W, Zhou C, Huang J. CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. *Protein Cell* 2015; **6**: 363-372 [PMID: 25894090 DOI: 10.1007/s13238-015-0153-5]

153 **Cyranoski D**, Ledford H. Genome-edited baby claim provokes international outcry. *Nature* 2018; **563**: 607-608 [PMID: 30482929 DOI: 10.1038/d41586-018-07545-0]

154 **Lander ES**, Baylis F, Zhang F, Charpentier E, Berg P, Bourgain C, Friedrich B, Joung JK, Li J, Liu D, Naldini L, Nie JB, Qiu R, Schoene-Seifert B, Shao F, Terry S, Wei W, Winnacker EL. Adopt a moratorium on heritable genome editing. *Nature* 2019; **567**: 165-168 [PMID: 30867611 DOI: 10.1038/d41586-019-00726-5]

155 **Ihry RJ**, Worringer KA, Salick MR, Frias E, Ho D, Theriault K, Kommineni S, Chen J, Sondey M, Ye C, Randhawa R, Kulkarni T, Yang Z, McAllister G, Russ C, Reece-Hoyes J, Forrester W, Hoffman GR, Dolmetsch R, Kaykas A. p53 inhibits CRISPR-Cas9 engineering in human pluripotent stem cells. *Nat Med* 2018; **24**: 939-946 [PMID: 29892062 DOI: 10.1038/s41591-018-0050-6]

156 **Merkle FT**, Ghosh S, Kamitaki N, Mitchell J, Avior Y, Mello C, Kashin S, Mekhoubad S, Ilic D, Charlton M, Saphier G, Handsaker RE, Genovese G, Bar S, Benvenisty N, McCarroll SA, Eggan K. Human pluripotent stem cells recurrently acquire and expand dominant negative P53 mutations. *Nature* 2017; **545**: 229-233 [PMID: 28445466 DOI: 10.1038/nature22312]

157 **Champer J**, Chung J, Lee YL, Liu C, Yang E, Wen Z, Clark AG, Messer PW. Molecular safeguarding of CRISPR gene drive experiments. *Elife* 2019; 8 [PMID: 30666960 DOI: 10.7554/eLife.41439]

158 **Gantz VM**, Jasinskiene N, Tatarenkova O, Fazekas A, Macias VM, Bier E, James AA. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi. *Proc Natl Acad Sci U S A* 2015; **112**: E6736-E6743 [PMID: 26598698 DOI: 10.1073/pnas.1521077112]

159 **Hammond A**, Galizi R, Kyrou K, Simoni A, Siniscalchi C, Katsanos D, Gribble M, Baker D, Marois E, Russell S, Burt A, Windbichler N, Crisanti A, Nolan T. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae. *Nat Biotechnol* 2016; **34**: 78-83 [PMID: 26641531 DOI: 10.1038/nbt.3439]

160 **The Lancet**. Safeguarding the future of human gene editing. *Lancet* 2017; **389**: 671 [PMID: 28229861 DOI: 10.1016/S0140-6736(17)30389-6]

161 **Khurshid AT**. Safeguarding the Impacts of Human Genome Editing. *Adv Biotech and Micro* 2018; 9 [DOI: 10.19080/AIBM.2018.09.555775]

162 **Otieno MO**. CRISPR-Cas9 Human Genome Editing: Challenges, Ethical Concerns and Implications. *Journal of Clinical Research and Bioethics* 2015; 6 [DOI: 10.4172/2155-9627.1000253]

163 **Zhou M**, Hu Z, Qiu L, Zhou T, Feng M, Hu Q, Zeng B, Li Z, Sun Q, Wu Y, Liu X, Wu L, Liang D. Seamless Genetic Conversion of SMN2 to SMN1 via CRISPR/Cpf1 and Single-Stranded Oligodeoxynucleotides in Spinal Muscular Atrophy Patient-Specific Induced Pluripotent Stem Cells. *Hum Gene Ther* 2018; **29**: 1252-1263 [PMID: 29598153 DOI: 10.1089/hum.2017.255]

164 **Calvo-Garrido J**, Maffezzini C, Schober FA, Clemente P, Uhlin E, Kele M, Stranneheim H, Lesko N, Bruhn H, Svenningsson P, Falk A, Wedell A, Freyer C, Wredenberg A. SQSTM1/p62-Directed Metabolic Reprogramming Is Essential for Normal Neurodifferentiation. *Stem Cell Reports* 2019; **12**: 696-711 [PMID: 30827875 DOI: 10.1016/j.stemcr.2019.01.023]

165 **Dong Y**, Peng T, Wu W, Tan D, Liu X, Xie D. Efficient introduction of an isogenic homozygous mutation to induced pluripotent stem cells from a hereditary hearing loss family using CRISPR/Cas9 and single-stranded donor oligonucleotides. *J Int Med Res* 2019; **47**: 1717-1730 [PMID: 30819013 DOI: 10.1177/0300060519829990]

166 **Sabol RA**, Bunnell BA. Discussion: CRISPR/Cas9-Mediated BRCA1 Knockdown Adipose Stem Cells Promote Breast Cancer Progression. *Plast Reconstr Surg* 2019; **143**: 757-758 [PMID: 30817647 DOI: 10.1097/PRS.0000000000005391]

167 **Yanagihara Y**, Inoue K, Saeki N, Sawada Y, Yoshida S, Lee J, Iimura T, Imai Y. Zscan10 suppresses osteoclast differentiation by regulating expression of Haptoglobin. *Bone* 2019; **122**: 93-100 [PMID: 30771488 DOI: 10.1016/j.bone.2019.02.011]

168 **van de Vrugt HJ**, Harmsen T, Riepsaame J, Alexantya G, van Mil SE, de Vries Y, Bin Ali R, Huijbers IJ, Dorsman JC, Wolthuis RMF, Te Riele H. Effective CRISPR/Cas9-mediated correction of a Fanconi anemia defect by error-prone end joining or templated repair. *Sci Rep* 2019; **9**: 768 [PMID: 30683899 DOI: 10.1038/s41598-018-36506-w]

169 **Blanas A**, Cornelissen LAM, Kotsias M, van der Horst JC, van de Vrugt HJ, Kalay H, Spencer DIR, Kozak RP, van Vliet SJ. Transcriptional activation of fucosyltransferase (FUT) genes using the CRISPR-dCas9-VPR technology reveals potent N-glycome alterations in colorectal cancer cells. *Glycobiology* 2019; **29**: 137-150 [PMID: 30476078 DOI: 10.1093/glycob/cwy096]

170 **Sun T**, Patil R, Galstyan A, Klymyshyn D, Ding H, Chesnokova A, Cavenee WK, Furnari FB, Ljubimov VA, Shatalova ES, Wagner S, Li D, Mamelak AN, Bannykh SI, Patil CG, Rudnick JD, Hu J, Grodzinski ZB, Rekechenetskiy A, Falahatian V, Lyubimov AV, Chen YL, Leoh LS, Daniels-Wells TR, Penichet ML, Holler E, Ljubimov AV, Black KL, Ljubimova JY. Blockade of a Laminin-411-Notch Axis with CRISPR/Cas9 or a Nanobioconjugate Inhibits Glioblastoma Growth through Tumor-Microenvironment

Cross-talk. *Cancer Res* 2019; **79**: 1239-1251 [PMID: 30659021 DOI: 10.1158/0008-5472.CAN-18-2725]
171 **Jelinkova S**, Fojtik P, Kohutova A, Vilotic A, Marková L, Pesl M, Jurakova T, Kruta M, Vrbsky J, Gaillyova R, Valášková I, Frák I, Lacampagne A, Forte G, Dvorak P, Meli AC, Rotrekl V. Dystrophin Deficiency Leads to Genomic Instability in Human Pluripotent Stem Cells via NO Synthase-Induced Oxidative Stress. *Cells* 2019; 8 [PMID: 30650618 DOI: 10.3390/cells8010053]
172 **Hurtado Del Pozo C**, Garreta E, Izpisúa Belmonte JC, Montserrat N. Modeling epigenetic modifications in renal development and disease with organoids and genome editing. *Dis Model Mech* 2018; 11 [PMID: 30459215 DOI: 10.1242/dmm.035048]
173 **Tang L**, Yao F, Wang H, Wang X, Shen J, Dai B, Wu H, Zhou D, Guo F, Wang J, Li T, Wang H, Gong T, Su J, Wang L, Liang P. Inhibition of TRPC1 prevents cardiac hypertrophy via NF-κB signaling pathway in human pluripotent stem cell-derived cardiomyocytes. *J Mol Cell Cardiol* 2019; **126**: 143-154 [PMID: 30423318 DOI: 10.1016/j.yjmcc.2018.10.020]
174 **Tian L**, Ye Z, Kafka K, Stewart D, Anders R, Schwarz KB, Jang YY. Biliary Atresia Relevant Human Induced Pluripotent Stem Cells Recapitulate Key Disease Features in a Dish. *J Pediatr Gastroenterol Nutr* 2019; **68**: 56-63 [PMID: 30358741 DOI: 10.1097/MPG.0000000000002187]
175 **Wang S**, Liu Z, Ye Y, Li B, Liu T, Zhang W, Liu GH, Zhang YA, Qu J, Xu D, Chen Z. Ectopic hTERT expression facilitates reprograming of fibroblasts derived from patients with Werner syndrome as a WS cellular model. *Cell Death Dis* 2018; **9**: 923 [PMID: 30206203 DOI: 10.1038/s41419-018-0948-4]
176 **Barnes J**, Salas F, Mokhtari R, Dolstra H, Pedrosa E, Lachman HM. Modeling the neuropsychiatric manifestations of Lowe syndrome using induced pluripotent stem cells: defective F-actin polymerization and WAVE-1 expression in neuronal cells. *Mol Autism* 2018; **9**: 44 [PMID: 30147856 DOI: 10.1186/s13229-018-0227-3]
177 **Frasier CR**, Zhang H, Offord J, Dang LT, Auerbach DS, Shi H, Chen C, Goldman AM, Eckhardt LL, Bezzerides VJ, Parent JM, Isom LL. Channelopathy as a SUDEP Biomarker in Dravet Syndrome Patient-Derived Cardiac Myocytes. *Stem Cell Reports* 2018; **11**: 626-634 [PMID: 30146492 DOI: 10.1016/j.stemcr.2018.07.012]
178 **Sasaki-Honda M**, Jonouchi T, Arai M, Hotta A, Mitsuhashi S, Nishino I, Matsuda R, Sakurai H. A patient-derived iPSC model revealed oxidative stress increases facioscapulohumeral muscular dystrophy-causative DUX4. *Hum Mol Genet* 2018; **27**: 4024-4035 [PMID: 30107443 DOI: 10.1093/hmg/ddy293]
179 **Wang H**, Guo R, Du Z, Bai L, Li L, Cui J, Li W, Hoffman AR, Hu JF. Epigenetic Targeting of Granulin in Hepatoma Cells by Synthetic CRISPR dCas9 Epi-suppressors. *Mol Ther Nucleic Acids* 2018; **11**: 23-33 [PMID: 29858058 DOI: 10.1016/j.omtn.2018.01.002]
180 **Moghaddas F**, Zeng P, Zhang Y, Schützle H, Brenner S, Hofmann SR, Berner R, Zhao Y, Lu B, Chen X, Zhang L, Cheng S, Winkler S, Lehmberg K, Canna SW, Czabotar PE, Wicks IP, De Nardo D, Hedrich CM, Zeng H, Masters SL. Autoinflammatory mutation in NLRC4 reveals a leucine-rich repeat (LRR)-LRR oligomerization interface. *J Allergy Clin Immunol* 2018; **142**: 1956-1967.e6 [PMID: 29778503 DOI: 10.1016/j.jaci.2018.04.033]
181 **Liu C**, Banister CE, Weige CC, Altomare D, Richardson JH, Contreras CM, Buckhaults PJ. PRDM1 silences stem cell-related genes and inhibits proliferation of human colon tumor organoids. *Proc Natl Acad Sci U S A* 2018; **115**: E5066-E5075 [PMID: 29760071 DOI: 10.1073/pnas.1802902115]
182 **Jiao J**, Tian W, Qiu P, Norton EL, Wang MM, Chen YE, Yang B. Induced pluripotent stem cells with NOTCH1 gene mutation show impaired differentiation into smooth muscle and endothelial cells: Implications for bicuspid aortic valve-related aortopathy. *J Thorac Cardiovasc Surg* 2018; **156**: 515-522.e1 [PMID: 29653750 DOI: 10.1016/j.jtcvs.2018.02.087]
183 **Lyu C**, Shen J, Wang R, Gu H, Zhang J, Xue F, Liu X, Liu W, Fu R, Zhang L, Li H, Zhang X, Cheng T, Yang R, Zhang L. Targeted genome engineering in human induced pluripotent stem cells from patients with hemophilia B using the CRISPR-Cas9 system. *Stem Cell Res Ther* 2018; **9**: 92 [PMID: 29625575 DOI: 10.1186/s13287-018-0839-8]
184 **Deng WL**, Gao ML, Lei XL, Lv JN, Zhao H, He KW, Xia XX, Li LY, Chen YC, Li YP, Pan D, Xue T, Jin ZB. Gene Correction Reverses Ciliopathy and Photoreceptor Loss in iPSC-Derived Retinal Organoids from Retinitis Pigmentosa Patients. *Stem Cell Reports* 2018; **10**: 2005 [PMID: 29874627 DOI: 10.1016/j.stemcr.2018.05.012]
185 **Wattanapanitch M**, Damkham N, Potirat P, Trakarnsanga K, Janan M, U-Pratya Y, Kheolamai P, Klincumhom N, Issaragrisil S. One-step genetic correction of hemoglobin E/beta-thalassemia patient-derived iPSCs by the CRISPR/Cas9 system. *Stem Cell Res Ther* 2018; **9**: 46 [PMID: 29482624 DOI: 10.1186/s13287-018-0779-3]
186 **Suda Y**, Kuzumaki N, Sone T, Narita M, Tanaka K, Hamada Y, Iwasawa C, Shibasaki M, Maekawa A, Matsuo M, Akamatsu W, Hattori N, Okano H, Narita M. Down-regulation of ghrelin receptors on dopaminergic neurons in the substantia nigra contributes to Parkinson's disease-like motor dysfunction. *Mol Brain* 2018; **11**: 6 [PMID: 29458391 DOI: 10.1186/s13041-018-0349-8]

Published By Baishideng Publishing Group Inc
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
Telephone: +1-925-2238242
E-mail: bpgoffice@wjgnet.com
Help Desk: https://www.f6publishing.com/helpdesk
https://www.wjgnet.com