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CRISPR AND HUMAN APPLICATIONS

Alexander Einschütz López

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Introduction

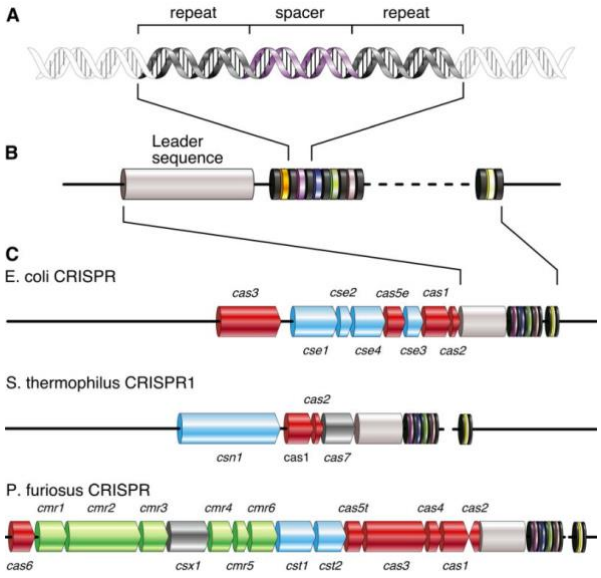
- History

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 technology is a molecular tool used to "edit" or "correct" the genome of any cell. It is usually described as a molecular scissor capable of cutting the DNA or RNA in a very precise and controlled way. The ability of CRISPR to cut the DNA and RNA is what permits the modification of its sequence by removing or inserting new segments.

The trail to the discovery of CRISPR began when Ishino et al. (1987), published an article describing a nucleotide sequence of *Escherichia coli.* Later, Mojica et al. (1993) described a very similar nucleotide sequence but it was in archaea. Finally, Mojica et al. (2000) described the biological significance of a family of regularly spaced repeats in the genomes of archaea, bacteria, and mitochondria. Jansen et al. (2002), started to refer these repeats as CRISPR an acronym used for Clustered Regularly Interspaced Short Palindromic Repeats.

However, it was not until 2007 when Barrangou et al. (2007) could prove that the CRISPR-Cas complex is a prokaryotic acquired immune system.

In 2012, Jinek et al. (2012) published an article in the Science magazine that explained how to turn the CRISPR prokaryotic immune system into a "programmable" editing tool. Jinek et al.(2012) were able to program the CRISPR system to target a specific location of any DNA (not just viral) and cleave it, thereby creating a cheap and fast genome editing tool.



(Karginov & Hannon, 2010)

- Mechanism of Action of CRISPR

When a virus infects a bacterium, in order to complete its life cycle, it takes control over its cellular machinery and interacts with it. However, bacteria to protect themselves, use the CRISPR-Cas9 defence system to form a complex composed of a bacterial Cas protein linked to the RNA produced from the CRISPR sequences which in turn, interact with viral genetic material. When this happens, the invasive virus is inactivated and subsequently degraded. (Yoshizumi Ishino et al., 2018)

Nonetheless, the CRISPR system has other functions where the Cas proteins take a small part of the viral DNA, which is then modified and integrated into the CRISPR sequence. The capacity to integrate viral DNA enables the bacterium (or its offspring) to inactivate the viral genetic material more efficiently if it encounters the same virus. (Yoshizumi Ishino et al., 2018)

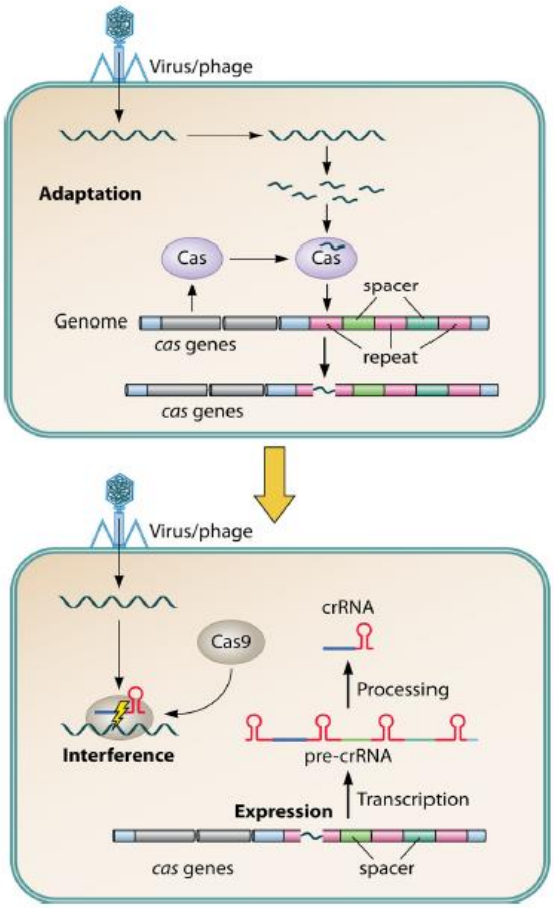


FIG 4 Process of CRISPR-Cas acquired immune system. (Top) Adaptation. The invading DNA is recognized by Cas proteins, fragmented and incorporated into the spacer region of CRISPR, and stored in the genome. Expression (bottom). Pre-crRNA is generated by transcription of the CRISPR region and is processed into smaller units of RNA, named crRNA. (Bottom) Interference. By taking advantage of the homology of the spacer sequence present in crRNA, foreign DNA is captured, and a complex with Cas protein having nuclease activity cleaves DNA.

(Yocshizumi Ishino et al., 2018)

To use this immune system as a programable editing tool that is able to cut a specific position of DNA, it is necessary to design a guide RNA, which will then be transfected into a cell together with the CRISPR-Cas9 complex. Inside the nuclei, the guide will find the homology sequence of the genome, it will hybridize and become the recognition signal for the Cas9 to cleave the DNA. (El-Mounadi et al., 2020)

The process of genome editing with CRISPR-Cas9 has two steps. The first step is an attack made by the RNA guide associated with the Cas9 enzyme. The RNA guide is unique to a distinct DNA sequence homology, based on nucleotide complementarity, this guide will hybridize to the target sequence (the region of the genome we are interested in editing or correcting). Then Cas9 acts, which is an endonuclease enzyme (a protein that is capable of breaking a bond in the chain of nucleic acids), causing a DNA double strand break. (El-Mounadi et al., 2020)

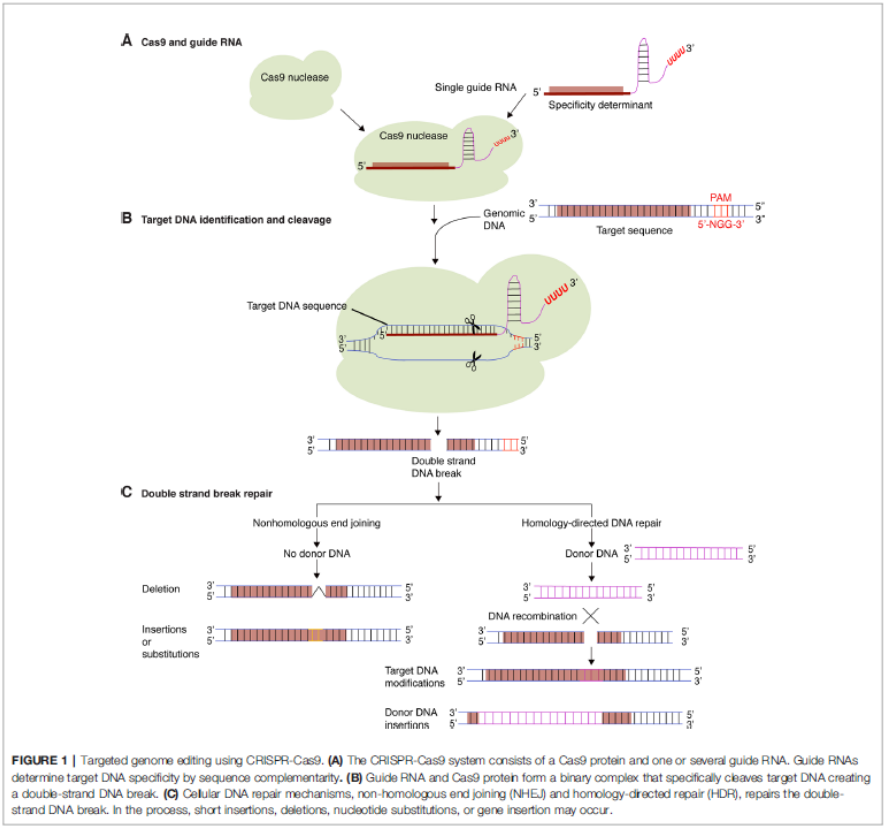


FIGURE 1 | Targeted genome editing using CRISPR-Cas9. **(A)** The CRISPR-Cas9 system consists of a Cas9 protein and one or several guide RNA. Guide RNAs determine target DNA specificity by sequence complementarity. **(B)** Guide RNA and Cas9 protein form a binary complex that specifically cleaves target DNA creating a double-strand DNA break. **(C)** Cellular DNA repair mechanisms, non-homologous end joining (NHEJ) and homology-directed repair (HDR), repairs the double-strand DNA break. In the process, short insertions, deletions, nucleotide substitutions, or gene insertion may occur.

(El-Mounadi et al., 2020)

In the second step, at least two natural DNA repair mechanisms are activated. The first one is the nonhomologous end-joining (NHEJ), that causes an indel (insertion-deletion) in the cleavage site (the homology DNA region were the RNA guide hybridize). The repair of this cleavage can cause a loss of DNA sequence or an insertion of novel DNA

sequence. Both events can lead to a loss of the DNA reading frame and as a consequence the knockout of genes. (El-Mounadi et al., 2020)

The second repair mechanism is the homologous-directed DNA repair (HDR), which allows the incorporation of a specific homology sequence, that replaces with a *bona fide* the original cleavage site. To allow this mechanism to occur we must introduce into the cell the homology sequence that we want to integrate into the DNA. (El-Mounadi et al., 2020)

Applications

- General fields

The CRISPR-cas9 system has been available since 2012, and its discovery has had a massive impact in the World, with the potential to be applied in different areas including agriculture, medicine and biofuels.

Agriculture

Between 2015 and 2016 great advances were made in the agrotechnology field, where non-browning apples, non-browning mushrooms and reduced bruising and browning of potatoes were accepted in the market since they were not regulated by the USDA (U.S. DEPARTMENT OF AGRICULTURE). The idea behind these discoveries is to reduce food waste and to prolong the shelf-life of other perishable foods. (Waltz, 2015a, 2015b, 2016)

Another big improvement in the agrotechnology field came with the idea of using CRISPR to enable crop survival under rough conditions. A clear example of this is represented by the tomato. Since 2014 the tomato has been undergoing four kinds of major modifications in order to improve its quality, its resistance to biotic stresses and to abiotic stresses and its domestication (controlling the seed size, dispersal mechanism and timing of germination, flowering and ripening). (Wang et al., 2019)

Medicine

In the field of medicine, CRISPR technology has had a great impact, allowing to develop rapid diagnostic tests to detect Viral and Bacterial RNA/DNA, and tumor DNA from body fluids. (Foss et al., 2019)

he properties of Cas12 and Cas13a proteins, target specifity and trans cleavage activity have positively impacted in the capacity of CRISPR application in diagnosis. The benefits of using CRISPR-based detection test lies in its speed, accuracy, and the fact that it is very affordable. (Chen et al., 2017; East-Seletsky et al., 2016)

Some companies have already some products based on the CRISPR technology in the market. This is the case of Sherlock Biosciences Corp. (Boston, MA) which has already the Sherlock™ CRISPR SARS-CoV-2 kit, a diagnostic kit based on CRISPR-Cas13a technology to detect SARS CoV-2. This kit has not been approved by the U.S. Food and Drug Administration (FDA). But due to the emergency state caused by SARS-CoV-2 pandemic, the FDA authorized its use under an Emergency Use Authorization (EUA) issued to Sherlock Biosciences. (*Sherlock CRISPR SARS-CoV-2 Kit* | *IDT*, n.d.)

Another big revolution is the fact that we can now treat some uncurable diseases, there are studies in animal models that showed results for a lot of hereditable and non-hereditable genetic diseases, the most interesting one being sickle cell anemia (Paganelli, 2019)

Biofuels

In the field of biofuels, the use of CRISPR to genetically modify bacteria and algae has opened the door to the third generation of biofuels.

At the University of California, Ajjawi et al. (2017) have used CRISPR to knockout a homolog of fungal Zn(ii)2Cys6-encoding genes that resulted in doubling the lipid production in algae. This research was funded by both synthetic genomics and the ExxonMobile companies with the aim to enhance the production of biofuels. (*ExxonMobil and Synthetic Genomics Algae Biofuels Program Targets 10,000 Barrels Per Day by 2025* | *Synthetic Genomics, Inc.*, n.d.)

There are also some studies in *Clostridium autoethanogenum* that show an improved efficiency of gene knock-out organisms in the production of biogas. (Nagaraju et al., 2016)

- Pathological model creation

To reach the market, novel drugs and treatments must first pass several preclinical and clinical studies in where they have to show they are safe, effective and has no or limited side effects. Animal tests constitute one of the first preclinical studies in order to estimate how safe it is for a living being and which side effects it may have.

Until recently, most of the animal models used to perform these preclinical studies needed several generations of germline editing in order to obtain a living organism that presented a specific disease. Since the discovery of CRISPR as a genome editing tool, it is possible to obtain one-step generation of mammals carrying mutations that cause the different types of diseases in multiple genes (Li et al., 2019)

There are four ways of creating pathologic models, the first one is by creating site-precise alterations in embryonic stem cells that later are going to be introduced into blastocysts. The second way is to inject the editing elements into the zygotes. The third way is to edit

somatic cell genomes and transferring them into a zygote using somatic cell nuclear transfer technology. And the last way, is to introduce engineered viruses loaded with editing elements into adult animals (Li et al., 2019)

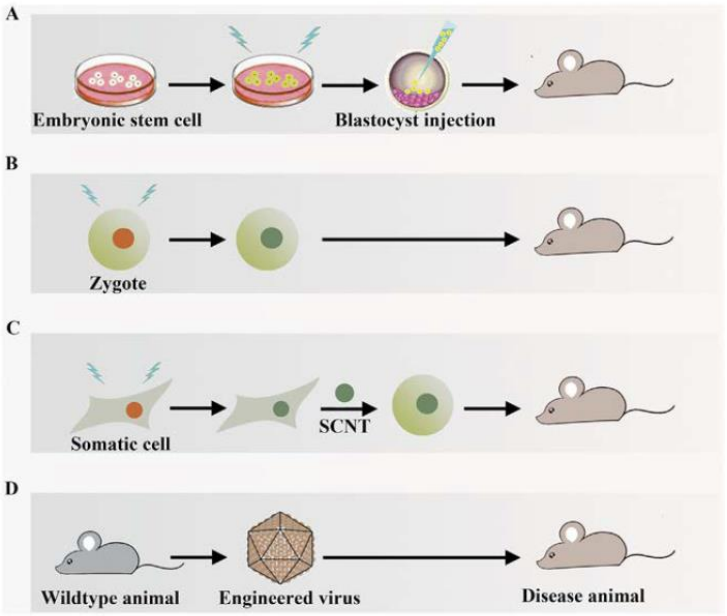


Fig. 2. Schematic overview of constructing disease animal model in four major ways. A) Cultured embryonic stem cells (ESCs) can be used to introduce morbigenous mutations using genome editing tools. The edited ESCs can be injected into host blastocysts, whereafter are implanted into pseudo-pregnant to produce disease animal; B) Animal zygote is directly edited and the edited zygote is developed into diseased model; C) Disease animal could be generated by combining somatic cell genome editing and somatic cell nuclear transfer (SCNT) technology; D) Genome editing elements are packaged by viral vectors. Disease animal can be generated by administration of engineered virus.

(Li et al., 2019)

There are already a variety of studies that show the use of the CRISPR-Cas9 complex in order to successfully obtain a pathological model for cancer (Antal et al., 2015; Chiou et al., 2015; Loayza-Puch et al., 2016; Platt et al., 2014; Sanchez-Rivera et al., 2014), cardiovascular diseases (K. J. Carroll et al., 2016; Huang et al., 2017), ophthalmic diseases (Yuan et al., 2016; Zhong et al., 2015), metabolic diseases (Jiang et al., 2018; Turer et al., 2018), and neuropathic and muscle diseases (Sui et al., 2018; Yan et al., 2018).

Cancer models

In a study performed by Sanchez-Rivera et al. (2014) the authors edited the *KRAS* and *p53* tumor suppressor genes using the CRISPR-Cas9 complex. The outcome of these suppression resulted in the generation of lung adenocarcinome in mice 10 weeks after infection.

A similar experiment was done by Platt et al. (2014) in where they administered through both intranasal and intratracheal methods adeno-associated virus (AAV-9) carrying the CRISPR-Cas9 complex plus the vectors for the *KRAS*, *p53*, and *LKB1* tumor suppressor

genes. Two months later, the average total tumor burden was close to 10% of the total lung volume. Over time, the size of the tumors and the total tumor area significantly increased. The complexity of the formation and evolution of the tumors resembles the one observed in human lung cancer.

There are also models for liver cancer (Xue et al., 2014), breast carcinoma (Loayza-Puch et al., 2016), pancreatic cancer (Chiou et al., 2015), and colon cancer (Antal et al., 2015).

Cardiovascular models

In the cardiovascular field, Carroll et al. (2016) achieved a hypertrophic cardiomyopathy in a transgenic mouse. In order to avoid embryonic lethality, they created transgenic mice by injecting Cas9 expression plasmids that are regulated by the Myh6 promoter in mouse zygotes. Afterwards they used the Adeno-Associated Virus 9 (AAV9) to deliver encoding sgRNA against Myh6, resulting in the edition of the Myh6 locus in the cardiomyocytes. The transgenic mice presented severe cardiomyopathy and loss of cardiac function, with increased heart failure markers five weeks after the AAV9 delivery.

Until recently, most of the cardiovascular disease models were done in mice, but there is a large difference in the cardiovascular system between mice and humans. Pig models have been developed since their cardiovascular system is more analogue to the one of humans. One of such models has been archived by Huang et al. (2017).

Atherosclerosis is mainly caused by an unbalance of lipids in blood, especially high levels of low-density lipoproteins (LDL). Since *ApoE* and *LDLR* gene mutations play a large role in this disease evolution, Huang et al. (2017) modified pigs using the CRISPR-Cas9 technology. Their team targeted the *ApoE* and *LDLR* genes in pigs, obtaining biallelic knockout pigs.

These mutant pigs exhibited significantly higher levels of low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and apolipoprotein B (APOB) than normal pigs. This abnormal lipid metabolism related to atherosclerosis animal models will be a valuable model to test novel therapies to treat human cardiovascular diseases.

Ophthalmic models

Since there are multiple hereditary eye diseases with ocular manifestations that involve the various parts of the eye (cornea, iris, retina, etc.), it has been a growing interest in generating animal models that recapitulate the pathological features of these disorders. The pathophysiology of many ophthalmic diseases is mostly unknown, and as a consequence, treatments have little or no effectiveness. The lack of treatments exerts a negative influence on patient's vision and quality of life. (Cabral et al., 2017)

The primary ophthalmic disease that causes loss of vision in humans is the development of cataracts. Even though cataracts can be treated with surgical methods, there is a need to construct a suitable animal model to study the safety of those surgical methods and its interaction with drugs and medications.

Yuan et al. (2016) , co-injected the CRISPR-Cas9 complex, together with the *GJA8* gene guide RNA (sgRNA) into rabbit zygotes. All the pups were born with mutations in the *GJA8* edited gene. Impaired *GJA8* function caused microphthalmia, small lens size, and cataracts, which provided a good disease model for cataract related research.

Leber's congenital amaurosis is an ophtalmic disease that appears at birth or during the first few months of life. It causes early blindness due to mutations in one of the multiple associated genes, depending on which gene is mutated, it causes one of the 18 known types. Leber's congenital amaurosis type 16 is related to the KCNJ13.

Zhong et al. (2015) injected the CRISPR-Cas9 complex to mouse embryos, producing KCNJ13 mutants. The complete loss of KCNJ13 expression is postnatal lethal. Among the surviving KCNJ13 mutants, 80% showed mosaicism in the expression of retinal pigment epithelium. Mosaicism occurs when an individual presents two type of cells, mutant or engineered type and the wild or normal type. This mosaicism is associated with a decreased response to light and photoreceptor degradation. The establishment of this model could mimic human Leber's congenital amaurosis type 16 disease.

Metabolic models

Every living being is always metabolizing, so any kind of metabolic abnormality can lead to a great variety of diseases. Obesity is nowadays one of the disorders with higher incidence in Western countries.

Turer et al. (2018) researching genetic causes of obesity, detected a correlation of obesity with a mutation in the *ARNT2* gene. In order to demonstrate this correlation, they used the CRISPR-Cas9 complex to target the Arnt2 gene, obtaining a single-nucleotide replacement allele (C→T at chromosome 7 q.84,347,530).

The Arnt2 mutant mice showed hyperphagic obesity, diabetes, and hepatic steatosis. This animal model proved the role of Arnt2 in obesity and became a pathological model to test treatments.

Wilson disease is a rare metabolic disease caused by accumulation of copper metabolism in the liver. This disorder is due to mutations in the *ATP7B* gene, which translates for a protein that regulates the transmembrane transport of copper in hepatocytes.

Jiang et al. (2018) used the CRISPR-Cas9 complex to create an ATP7B mutant rabbit model. These mutant rabbits showed a nine-fold increase of copper in their livers, these

values were reminiscent of those found in patients with Wilson disease. These results proved to be a potential good model of Wilson disease.

Neuropathic and muscle diseases

Neurodegenerative diseases have become fore front in being depicted as some of the worst diseases nowadays. For most of them there are no accurate diagnoses or effective treatment, and their prevalence is increasing as human life expectancy raises.

Huntington disease is a progressive brain disorder that is characterized by the loss of medium spiny neurons in the striatum. The loss of these neurons causes uncontrolled movements, emotional problems, and loss of cognitive abilities.

Yan et al. (2018) in order to create a Huntington disease model, applied the CRISPR-Cas9 complex in pigs. They inserted the human Huntington's mutation gene containing the 150CAG repeat into the Huntington gene locus.

The mutant pigs replicated the movement disorder, they evidenced behavioural abnormalities, and had shorter life sans compared to wild-type pigs. After further investigations, they also showcased the selective degeneration of striatal medium spiny neurons, making them the first large animal model for neurodegenerative diseases. (Yan et al., 2018)

Duchenne muscular dystrophy is a very common muscular dystrophy, with an incidence of 1 of every 3500 new male births. This disease is caused by a mutation in the dystrophin gene located at the X chromosome. Mice models of Duchenne muscular dystrophy already exist, but do not faithfully recreate the Duchenne muscular dystrophy shown in patients due to their small size and the limitations on chronic muscular lesions and muscle weakness.

Taking all of this into consideration, Sui et al. (2018) produced a Duchenne muscular dystrophy rabbit model. They achieved this by injecting the CRISPR-Cas9 complex into rabbit zygotes, thereby obtaining Duchenne muscular dystrophy knockout rabbits. These knockout rabbits expressed the typical phenotypes of Duchenne muscular dystrophy such as impaired physical activity, elevated serum creatine kinase levels, and progressive muscle necrosis and fibrosis. When the rabbits were 5 months old, the also showcased clear pathologies in the diaphragm and the hearth, like those patients afflicted with Duchenne muscular dystrophy.

Thus, what these researchers achieved was a novel Duchenne muscular dystrophy knockout rabbit model that recreates the hallmarks of the human diseases, being a good model for preclinical trials.

- Clinical diagnosis

SHERLOCK and DETECTR

In 2015 two new Cas proteins were discovered, Cas12a known as Cpf1 and Cas13a known as C2c2. (Shmakov et al., 2015; Zetsche et al., 2015)

The Cas13a protein was discovered by Shmakov et al. (2015)who described the Cas13a protein containing an effector with two predicted HEPN RNase domains. Abudayyeh et al. (2016) improved the description of the protein explaining that Cas13a is a "single-component programmable RNA-guided RNA-targeting CRISPR effector", and East-Seletsky et al. (2016) described the trans-cleaving activity of the cas13a protein and showed how to use it as a detection tool.

The trans-cleavage activity of Cas13a enables the detection of specific RNA sequences because after the recognition of the target RNA, Cas13a splits any nearby single strand RNA sequence acting like a shredder. If the nearby RNA sequences are reporters, having in one end of the sequence a fluorophore and in the other end a quencher, it can be used to detect the trans-cleavage activity of Cas13a by the presence of fluorescence.

In 2017 Gootenberg et al. (2017)used the Cas13a protein to create SHERLOCK (Specific High-Sensitivity Enzymatic Reporter unLOCKing), a diagnostic platform that uses the trans-cleavage property of the Cas13a protein to detect strains of Zika and Dengue virus. The system distinguish pathogenic bacteria and identify mutations in cell-free tumor DNA.

The Cas12a protein was discovered by Zetsche et al. (2015) and described as a two-component RNA programable DNA nuclease. Chen et al. (2017) described the trans-cleaving activity of Cas12a, explaining that once the DNA binding occurs, the Cas12a protein cleaves any nearby single-strand DNA. They also created a method called DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter) that uses the trans-cleavage activity of Cas12a to detect specific sequences of DNA. Using this method, they were able to detect human papillomavirus in patients, enabling so another platform for molecular diagnostics.

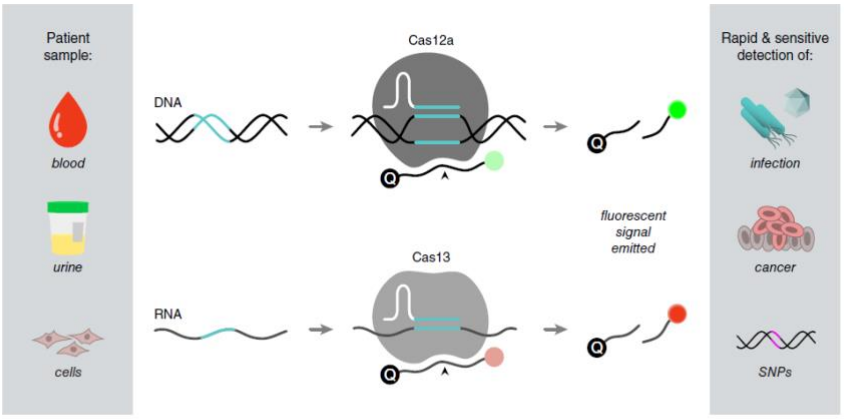


Fig. 3. Diagnostic applications of CRISPR-Cas enzymes. The ability to recognize precise genetic sequences allows use of CRISPR-Cas enzymes for biomedical testing. Cas12a (top) and Cas13 (bottom) can respectively be programmed to recognize DNA or RNA, activating the enzymes to cleave nearby nucleic acid molecules. Single-stranded DNA or RNA molecules bearing a fluorophore and a quencher (Q) are provided as part of the detection system, and these will result in a fluorescent signal following cleavage by Cas12a or Cas13, respectively. Because this signal will only result if the CRISPR-Cas enzyme finds a sequence matching its targeting RNA (cyan), this system can perform exquisitely sensitive detection of genetic sequences associated with pathogens or disease.

Foss et al., 2019

Sherlock Biosciences Corp. (Boston, MA) is currently selling Sherlock™ CRISPR SARS-CoV-2 Kits for 995$ USD each (around 823€ EUR). The Sherlock™ CRISPR SARS-CoV-2 Kit permits up to 33 tests per kit, and only needs approximately 1 hour per test. Mammoth Biosciences company published last year an article in nature biotechnology in where they used an DETECTR assay to detect SARS-CoV-2. (Broughton et al., 2020)

Both detection kits, SHERLOCK and DETECTR, are still under validation studies and have not been approved for diagnostics yet. But the FDA issued an Emergency Use Authorization (EUA) to Sherlock Biosciences to use their kit in authorized laboratories. (*Sherlock CRISPR SARS-CoV-2 Kit | IDT*, n.d.)

- Genetic engineering

After the discovery of the CRISPR technology, the treatment of genetic hereditable diseases has been under debate. Researchers around the globe, have been interested in using this mechanism to treat monogenic genetic diseases. Two kinds of approaches have been investigated using CRISPR: the somatic gene therapy and the germline therapy, being the germline therapy the most controversial.

Germline therapy

In 2015 the first human embryo was treated with the CRISPR-Cas9 complex, it was done by Liang et al. (2015) in order to increase the understanding of the CRISPR-Cas9 complex. They used CRISPR to cleave the endogenous β-globin gene in tripronuclear zygotes. Tripronuclear zygotes are egg cells that have been fecundated with two sperm cells and can develop up to blastocyte *in* vitro but fail to develop normally *in* vivo. The results showed a low efficiency of homologous recombination directed repair, producing

mosaic embryos. Furthermore, the endogenous delta-globin gene competed with the donor oligos to act as the template to repair the cleaved gene, provoking unwanted mutations. These results didn't discourage the scientists, it only displayed the need to improve the trustworthiness of the CRISPR-Cas9 complex in order to be used in clinical applications.

In 2016, Kang et al. (2016) used the CRISPR-Cas9 complex together with donor DNA to introduce CCR5Δ32, a beneficial and natural occurring allele, into the *CCR5* (C–C chemokine receptor type 5) gene. The CCR5Δ32 allele is an uncommon allele that is mainly present in Scandinavian and Northern European populations but is practically absent in non-European populations. The individuals who present this allele are healthy people that present a slower progression of HIV disease and an increased resistance to HIV infections.

The results they obtained were promising. Although the HDR repair mechanism had low efficiency, authors were able to introduce a beneficial allele into human tripronuclear zygotes using the CRISPR-Cas9 complex. They also did not detect any off-site targeting within the 28 predicted potential off-target sites, but since computational prediction based on sequence synonymity has shown to fail to predict off-targets, they could not discard the presence of off-target mutations in other unchecked regions of the genome.

Nevertheless, these results are encouraging and suggest that an optimised technique could be applied to correct disease-causing mutations in human zygotes.

In 2017 Ma et al. (2017) published an article in Nature where they treated a mutated heterozygous *MYBPC3* gene in human embryos with very high precision. The mutant *MYBPC3* gene causes hypertrophic cardiomyopathy, a disease with a prevalence of 1 every 500 people.

Mosaicism seen in human embryos treated with CRISPR-Cas9 could be explained mainly by two reasons: first is the completion of the cell cycle with DNA replication by the time the injection of the CRISPR-Cas9 occurs, causing only one of the two alleles to be modified, and the second reason is that the CRISPR-Cas9 complex remained active after the zygotic division, producing unwanted mutations.

To resolve the problem of mosaicism Ma et al. (2017) came up with a protocol that co-injects the CRISPR-Cas9 complex and the sperm into a M-phase oocyte. With this protocol, 42 out of 58 embryos were repaired via HDR, proving that injecting CRISPR–Cas9 into MII oocytes is more efficient than injecting it into zygotes. This protocol also prevents the presence of mosaicism. Authors also searched for off-targets performing a whole genome and exome sequencing and did not find any mutations.

Ma et al. (2017) used a unique zygotic DNA repair mechanism to eliminate mosaicism and off-targeting. But even though, 16 out of 58 (27.6%) zygotes were repaired through non-homologous end-joining (NHEJ). These results present an incredible advance in comparison to previous reports, but still are far away from reaching the required 95% correction needed for a treatment to be developed. Further research is needed to optimize the CRISPR technology to be applied to correct inheritable disorder in zygotes.

Somatic gene therapy

When working with somatic cells, researchers have two plausible approaches. The first one is an *ex* vivo approach, in where the cells are extracted from the body and after applying CRISPR, the engineered cells are returned to the body. The second approach is *in* vivo, where researchers introduce the CRISPR complex with a vector to modify specific cells to treat a disease.

The main approach used until now is ex vivo, due to the fact that it offers higher security, a broader range of delivery platforms, higher editing rates and control over the dosage. However, there are also some drawbacks being the most important one the fact that not all cell tissues can be used, since the target cells must be capable of surviving outside the body and that even after re-introduction cultured cells often engraft poorly, making the treatment less effective. (Cox et al., 2015)

In 2015 Park et al. (2015) used induced pluripotent stem cells (iPSCs) from a patient with haemophilia A, a genetic disorder provoked by mutations in the F8 gen. Almost half of the cases are due to chromosomal inversions. They corrected this chromosomal inversion editing the *F8* gene with the CRISPR-Cas9 complex. Afterwards the iPSCs were differentiated to endothelial cells expressing wild-type F8 gene without problems and without detectable off-target mutations. This successful experiment constitutes an starting point to establish a new treatment for haemophilia A.

In 2016 Dever et al. (2016) used for the first-time haematopoietic stem and Progenitor cells (HSPCs) to correct the Glu6Val mutation in the β-globin gene using the CRISPR-Cas9 complex together with an adeno-associated viral vector. This resulted in the correction of the mutation and the correct expression of adult β globin in erythrocytes after the corrected HSPCs differentiated. The correction of the Glu6Val mutation displayed the possibility of treating sickle cell anaemia using patient-derived stem and progenitor cells.

In order to develop a therapy for HIV-1, Xu et al. (2017) used CRISPR-Cas9 to knockout the *CCR5* gene in HSPCs. When the *CCR5* gene is absent, there is also a detectable loss of HIV-1 in the patient, so after treating the HSPCs and re-introducing them in mice the results obtained were favourable. The disruption efficiency remained robust in secondary haematopoietic cells and an HIV-1 resistance effect was observed. Xu et al. (2017) provided enough evidence to consider the edited HSPCs transplantation as an effective therapy to treat HIV-1.

The shortage of cancer-restricted surface markers constraints the use of the chimeric antigen receptor (CAR) T cells in immunotherapy. In acute myeloid leukemia (AML) the most common myeloid marker is the CD33 transmembrane receptor.

In 2018 Kim et al. (2018) used the CRISPR-Cas9 complex to generate *CD33* knocked out HSPCs. Mutants HSPCs did not express the CD33 transmembrane receptor and retained a normal myeloid function, enabling so the use of specific targeting of CD33 in AML with CAR T cells. In order to prove the efficiency of the CD33 knocked out HSPCs, these cells were tested in mice and non-human primates with AML. In both animal models

the leukemia was successfully eliminated, without myelotoxicity. These results generated a novel method for AML treatment using antigen-specific immunotherapy that avoided loss of normal myeloid cells.

The experimental data has provided until now very satisfying results in animal models. In October 2017, Dennis Normile published an article in Science explaining how 9 out of 10 clinical trials using CRISPR technology were in China and stating that China was getting ahead everybody else in this field. (Normile, 2017)

Bringing CRISPR into the clinic in China

Chinese researchers are pioneering the genome-editing tool CRISPR in human therapy. Their rapid advances are driven in part by a rising cancer burden and a paucity of experimental drugs when conventional treatments fail.

INSTITUTION	CONDITION	INTERVENTION	STATUS
Affiliated Hospital to Academy of Military Medical Sciences, Beijing	HIV infection	*CCR5* gene modification	Recruiting patients
First Affiliated Hospital of Sun Yat-sen University, Guangzhou	Cervical cancer	Disrupt human papillomavirus	In planning
Chinese PLA General Hospital, Beijing	Leukemia	Modified T cell receptors	Recruiting patients
Peking University, Beijing	Prostate cancer	PD-1 knockout T cells	In planning
Peking University, Beijing	Bladder cancer	PD-1 knockout T cells	In planning
Peking University, Beijing	Bladder cancer	PD-1 knockout T cells	In planning
Affiliated Nanjing Drum Tower Hospital of Nanjing University Medical School, Nanjing	Gastric and nasopharyngeal cancers	PD-1 knockout T cells	Recruiting patients
Hangzhou Cancer Hospital, Hangzhou	Esophageal cancer	PD-1 knockout T cells	Recruiting patients
Sichuan University, Chengdu	Lung cancer	PD-1 knockout T cells	Recruiting patients

Normile D., 2017

In 2018, the FDA granted permission to Vertex and CRISPR Therapeutics to begin clinical trials for CTX001, an investigational ex vivo CRISPR gene-edited therapy for patients suffering from transfusion dependent β-thalassemia or sickle cell disease. (Vertex Pharmaceuticals Incorporated, 2019)

The treatment consisted in the use of CRISPR to modify HSPC to produce higher levels of fetal haemoglobin to counter the effects of β-thalassemia and sickle cell disease.

The implantation of these edited cells in a patient affected of β-thalassemia had a significant impact: one year after the treatment, the patient did not require blood transfusions and his haemoglobin levels were 11.9g/dl (normal level: 13 g/dL for men and of 12 g/dL for women). The same treatment was also tested in a patient suffering from

sickle cell disease. The patient evidence a great improvement being free of vaso-occlusive crises with a total haemoglobin level of 11.3 g/dL. (Paganelli, 2019)

All the mentioned therapies until now were *ex* vivo therapies. But some *in* vivo therapies have also been developed. In September 2019 the U.S. Food and Drug Administration (FDA) approved the clinical trials of EDIT-101, an in vivo CRISPR therapy sponsored by Editas Medicine and Allergan, to treat Leber congenital amaurosis (LCA) type 10 caused by a point mutation in the CEP290 gene. (*Single Ascending Dose Study in Participants With LCA10 - Full Text View - ClinicalTrials.Gov*, n.d.)

This is the first in vivo trial using a CRISPR therapy, consisting in a subretinal injection of the EDIT-101 in a single eye. The expected results are an improvement in the vision capacity within four weeks, but initial results are still awaiting. ("First CRISPR Therapy Dosed," 2020)

There is also LUXTURNA (voretigene neparvovec-rzyl) a therapy that uses CRISPR to treat patients with Leber congenital amaurosis type 2. This treatment has been approved by the FDA and is used on patients with confirmed biallelic *RPE65* mutation. (*LUXTURNA | FDA*, n.d.)

This shows how CRISPR can be used to treat human diseases that until now were considered as untreatable. This is one of the first steps that will help to improve the number of clinical trials using this technique and the results, the generated data and the continuous research for novel therapies will help future researchers to make better procedures and to increase the number of treatable diseases.

Discussion

- Ethical concerns

The CRISPR-Cas9 technology as a tool to edit genes presents several advantages since its very simple, easy to use and cheap. It has been used to create pathological models for a wide range of diseases, to detect viral, bacterial and tumoral DNA and to treat some uncurable diseases until now. But the downsides of the technology had been the high off-target ratios, mosaicism, and unknown side effects.(Omodamilola & Ibrahim, 2018)

The problem of mosaicism can be solved using the procedure of Ma et al. where no mosaic cells were found, and the off-target and unknown side effects can be improved over time with continuous research, and new developing technologies like the base editors, transposases/recombinases and prime editors. Further research is still needed to solve these problems.

What really sparks conflict between different researchers is whether is ethical or not to use CRISPR in germline therapy. There is a major consensus in the use of CRISPR technology in research and as a therapy to correct somatic gene defects that causes

disease. Once you step out of this path and use CRISPR to edit germline cells you face big ethical problems, where the main arguments are the fear of designing babies, that germline editing violates the human dignity or that a generalized use of CRISPR will make the GATTACA film come true. (Beirain, 2018)

These are social concerns that coldly analysed are illogical. Right now we have not elucidated how most of the genes of our genome work and we recently found that almost all our phenotypes are caused by the interaction of different genes. It is true that we could engineer simple traits like the eye colour or the presence of freckles, but not complex traits such as height, intelligence or athletic capabilities.

There are already, to some extent, ways to choose simple traits, as is the case of the preimplantation genetic diagnosis (PGD), where in vitro fertilized embryos get their whole genome sequenced and parents can choose the embryo to be implanted. This is a current way to eliminate recessive traits from a family.

As for the appeal that CRISPR germline editing violates human dignity, there is an ease with which this concept is used despite its lack of specificity. Does respect for human dignity imply the need to preserve the genome as it is right now? And, if so, is this possible, when the genome is a perpetually changing reality? Furthermore, if we want to preserve the genome as it is, avoiding change, would this not rather imply a moral obligation to use CRISPR to reverse naturally produced mutations? These are hard to answer, therefore without further explanation on how CRISPR attacks human dignity it is a hard to accept argument.

At the end of 2018 a shocking new spread across the World, surprising researchers and everybody else evenly. In China a researcher called He Jiankui claimed that he made the world's first genetically edited babies using CRISPR. This new triggered people's concerns, being the greatest concern the health of the twin babies. (*Chinese Researcher Claims Birth of First Gene-Edited Babies - STAT*, n.d.; *"CRISPR Babies" Scientist He Jiankui Rose from Obscurity to Stun the World*, n.d.)

In previous studies, done by Carroll et al. (2016) there was embryonic lethality when there was an absence of a gene. This concern was soon mitigated by the news that twin babies Nana and Lulu were safely born. What outraged researchers was the fact that both babies presented mosaicism. He Jiankui and co-workers deleted the *CCR5* gene, in order to confer special resistance to HIV-1. Previous studies done by Kang et al. (2016) and Xu et al. (2017) showed that modified *CCR5* gene HSPCs could protect from the HIV-1 virus without any detectable off-targets. So what the researchers expect is that Nana and Lulu can have a good life. After this news, the social concerns for this technology were renewed.

Also, recent studies found out that CRISPR not only has off-targets and mosaicism as major problems, but also chromosomal alterations induced by on-target cleavage, the presence of anti-CRISPR proteins in phage and the activation of the p53 response to adeno-associated viral vectors causing a cell cycle arrest. (Pavani & Amendola, 2021)

- Potential work lines

There have been a lot of recent studies that gave hope for the clinical use of CRISPR, including both clinical trials for sickle cell disease and β -thalassemia (CTX001) and Leber congenital amaurosis type 10 (EDIT-101).

But there are still major challenges for many diseases. In a near future, it urge to dispose of good pathological models for neurodegenerative disease like Parkinson diseases, Alzheimer and multiple sclerosis, and also for neurodevelopmental disorders as schizophrenia or autism.

New CRISPR-based technologies have begun to appear, base editors are capable of changing one single letter in the DNA without provoking a doble strand break. This makes the reparation of the DNA more efficient and without off-target or on-target mutations (Anzalone et al., 2020). Right now, Base editors can change an adenosine (A) to a guanin (G) and a cytosine (c) to a thymine (T). This can help future researchers to develop new therapies that will help people that are suffering from cancer, neurofibromatosis (Serra et al., 2001) and Color blindness (J. Carroll et al., 2004) within others.

There are also good prospects for bone marrow cancer and Leukemia, since both of them may benefit of similar therapies to what has been used in the CTX001 treatment. Kim et al. (2018) showed how to use CRISPR to treat acute myeloid leukemia (AML) in mice and non-human primates.

To sum up, we are seeing continuous advances in the CRISPR technology that will enable researchers to keep improving and generating new therapies that can end up in clinical trials to treat human diseases that until now were untreatable. But we must not forget the fact that there are some major problems with the technique that need to be addressed before clinical trials begin and that, at least for now, germline therapies are going to be kept in the field of research until there is enough guarantee and safety for babies.

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