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APLICACION DE LA TECNOLOGIA CRISPR-CAS9 PARA CORREGIR DEFECTOS GENETICOS EN EMBRIONES

APPLICATION OF CRISPR-CAS9 GENOME EDITING TO CORRECT GENETIC DISORDERS IN EMBRYOS

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Abstract

The use of CRISPR has been a revolution in the field of the bioengineering. Not only is it allowing us to edit the human genome, but it also allows us to influence into the environment. In this paper we will focus on one of its most controversial applications, such as genetic edition in embryos. We will propose the possibility of treating Huntington's disease through this technique and we will briefly describe the problems that this type of approach faces.

Introduction

Huntington disease

Huntington disease (HD) is a hereditary neurodegenerative disorder that causes uncontrolled movements, emotional problems and loss of cognition ability. The primary etiology is a cortico-striatal pathology caused by a DNA triplet (CAG) expansion beyond 40 repetitions within the gen *Huntingtin* (*HTT*). It has a prevalence of approximately 3 to 7 individuals per 100,000 of European ancestry, being less common in other populations, such as Japanese, Chinese, and African descent.

The *HTT gene* is located into the chromosome 4p16.3 in a large locus, encompassing 180 kb and consisting of 67 exons (Fig. 1). The *HTT gene* is widely expressed and is required for normal neurological development. Usually it presents between 9 and 35 CAG repeats in the general population and an autosomal dominant inheritance. It is considered pathological when the CAG repeats get over 40. The repeated codons of CAG translate as polyglutamine repeats into the protein product.

Researches have shown that when there are less than 27 repeats, there is no manifestation of HD and there is stable transmission. In those cases where the repeat length is in the range of 27 to 35, there is no association with HD but there is a possibility of expansion upon transmission, and therefore genetic anticipation in descendants. This phenomenon entails that the next generations are most likely to present longer repetitions and an earlier symptoms onset, being the male transmission more prone to this to happen. The penetration is incomplete in cases in which the CAG repeats are in the range of 36 to 39, in which the presentation of the disease is variable.

The number of pathological CAG repeats in *HTT* has an inversely association with the disease onset, the larger the repeat-length the earlier the disease onset. Early onset associates with repeat-lengths between 50 and 60, while juvenile (JHD) onset associates with more than 60 repeats. Childhood onset is associated with more than 80 CAG repeats with the earliest diagnosis reported at 18-months of age, presenting more than 200 CAG repeats. Although the number of CAG repeats has a direct association with the age of onset, it seems not to have an impact in the course of the disease. This suggests that once the disease process begins, other factors determine its course.

The clinical symptoms of HD are classically defined by the triad of motor, cognitive and psychiatric alterations.

Motor symptoms and signs

Motor symptoms and signs are the first clinical manifestation that can be noticed at the onset, usually of small degree and distal localization.

Chorea is the milestone at the time of diagnosis. Characteristically it presents brief, abrupt, involuntary, non-stereotyped movements involving the trunk, face and limbs. In earlier stages the range of the movements are very moderate being even mistaken as restlessness. Furthermore, if the onset has only a focal motor sign, nearly half of the patients are unaware of them, even more they incorporate the chorea into purposeful movements. Other characteristics symptoms of the earlier stages are hypotonia, hyperreflexia, localized dystonia or mild bradykinesia. During the disease evolution the chorea becomes more flamboyant, to the point that it creates the impression of an outlandish dance, known in some places as "the dance of Saint Vito". In the late stages of the disease, motor function degenerates into a parkinsonian akinetic-rigid that makes walking unsafe.

In younger adult patients, abnormal eye movements can be found. There is absence of saccadic movement with preservation of smooth eye movements.

Cognitive dysfunction

The majority of patient presents some of this kind of symptoms. In earlier stages the presentation is rather limited to executive functions, difficulties in making decisions, multitasking, organization or planning. With the course of the disease other cognitive deficits emerge, until reaching the diagnosis of dementia. Even though it is a cortical dementia, it can be distinguished from other types of dementia by the lack of memory loss and cortical affection symptoms like aphasia, apraxia.

Psychiatric symptoms

Characteristically, the main symptoms are related to frontal lobe dysfunction. Earlier in the disease the frontal disinhibition with poor mood regulation, impulsivity, poor attention or irritability prevails. Family members usually describe it like a change in personality being the irritability the major concern because it turns into outbursts of anger and aggression. With the course of the disease the patient manifests symptoms of frontal abolition like apathy, loss of initiative, curiosity and creativity.

Depression is highly associated with HD. The timeline of the manifestations doesn't quite feet with the pathological disease substratum. The first period is around the diagnosis and the second period appears when function impairment begins to be more notable. Besides not been clear the etiology of the depression symptoms, it is most important to keep a tight control of the medication and a careful screening for suicide risk. With the course of the disease, the patients present a remarkable lack of awareness, a prominent feature of HD.

It is important to note, that depending on the time of onset, it may be some clinical differences. Adult forms can present cerebellar-type limb, gait ataxia, myoclonus,

seizures, or generalized dystonia, while JHD can present myoclonus, seizures, behavioral problems, and parkinsonism.

Importance of huntingtin protein

During this last decade, the huntingtin protein has become a major topic in terms of neurological development investigation. Recent research has shown that the *HTT* gene is highly conserved but, at the same time, the number of CAG repeats is not. Furthermore, it seems that the number of triplet repeats varies among species being the highest in humans. This supports a role in the transition of primates to humans.

Huntingtin protein expression is ubiquitous but the primary place where the disease settles down is the Striatum GABAergic spiny projection neurons (SPNs). Firstly, is affected the ISPNs indirect pathway (ISPNs) which oversee the inhibition of abnormal movements, their abolition causes the chorea. Over time, the direct route (dSPN) is affected and, as a result, bradykinesia appears, which is due to the fact that this type of neurons promotes appropriate movements.

Pre-HD patients have cerebral structural alterations and present some clinical manifestations even 20 years before the diagnosis. New theories posit that the triplets of CAG, generate an abnormal protein which accumulates into the neurons producing neurotoxicity and eventually the death of the cell. This leads to abnormal brain development which initially can be compensated but lately, gives way to the onset.

Gene therapy has a wild potential for treating this type of diseases. The two approaches that are followed now a day: First, restoring the health of the affected neurons and in this way recovering their correct function and normal pathway. Secondly, acting upon the gen to achieve neuroprotection slowing down the advance of the disease.
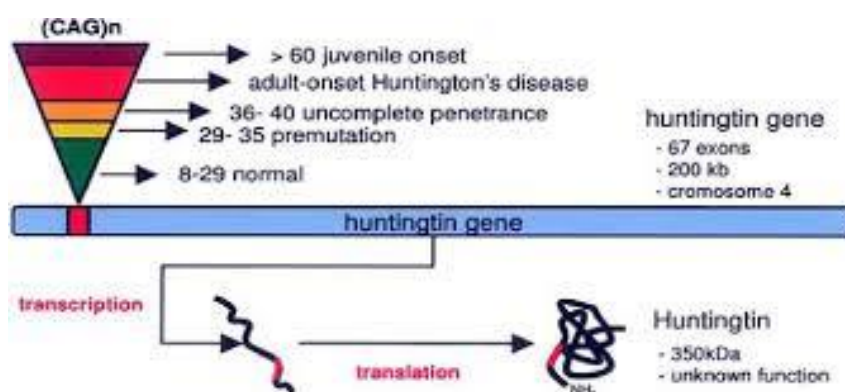

Figure 1

Schematic representation of the *HTT* gene. Reproduced from: Rego, A. and de Almeida, L. (2005). Molecular Targets and Therapeutic Strategies in Huntingtons Disease. *Current Drug Target -CNS & Neurological Disorders*, 4(4), pp.361-381.

CRISPR

Since the achievement of the human DNA sequencing with the Human genome project (HGP), researchers have proactively looked for new ways to use this information towards genetic engineering. In the last few years amazing discoveries and advances had been made in this field, but by far the most exciting one is the development of the CRISPR/Cas technology and the wide range of application it offers.

<u>Brief history and previous gene editors</u>

The gene edition era took off with the discovery of restriction enzymes during the late 70s. In the middle to late 80s, Capecchi and Smithies (6) demonstrated that some eukaryotic cells were able to introduce foreign genetic material into their own by using homologous recombination. This discovery gave the hint to the scientific community that maybe there was a mechanism that allowed to introduce exogenous genetic material into different cell types.

From that moment on, researchers started to focus on this new discovered system and tried to use it as a tool for genetic edition. While the advantages were very obvious, there also were some clear limits to it. The rate of spontaneous integration of an exogenous DNA was very low and it depended on the type and the status of the cell. Furthermore, it could generate unwanted integration into random genome loci at the same frecuency or higher that at the target site. With time, different approaches raise to solve this limits. One of the fist solutions that came along was the use of double strands break (DBS) at the target sites. The earlier attempts to achieve went through using rare cutting endo-meganuclease enzymes as I-Scel. This notoriously increased the frequency of target integration but generated other setbacks. Each meganuclease has a unique recognition sequence and the probability to target the specific loci was very low, not to mention that the majority of the cuts that were made by the DBS were repaired through the error-prone non-homologous end joining (NHEJ) DNA repair mechanism, which could result in random DNA integration or deletions (indels) at the break side. The researchers tried to overcome this new trammel by re-engineering naturally existing mega-nucleases to alter their DNA-targeting specificities with not much luck, only a small part of the genome could be specifically target in this way.

A breakthrough that helped further advance this field was the discovery of Zinc finger proteins. They are small zinc iron- regulated proteins motives which can bind with the DNA by recognized a specific sequence of 3-bp DNA sequence. Furthermore, as a difference of the endonuclease enzyme they could be ensembled into a large complex ($4^3\ combinations$) to achieve higher DNA binding specificity. With the discovery of the zinc finger domains, researches were able to create programmable nuclease proteins by merging zing fingers domains with the DNA cleavage domain of FOK I endonuclease (a restriction enzyme). Thanks to the ability of FOK I to homodimerize, zinc finger nucleases

(ZFNs) seems to increase targeted homologous recombination even in human and not only in model organisms. Soon after that, transcription activator-like effector (TALE) proteins were discovered. Like their predecessors, the zinc fingers, the TALE can be fused with Fok I DNA cleavage domain (TALEN), with the difference that TALE were able to specifically recognize one single base. This singularity allows TALEN to be an effective programmable nuclease (Fig 2).
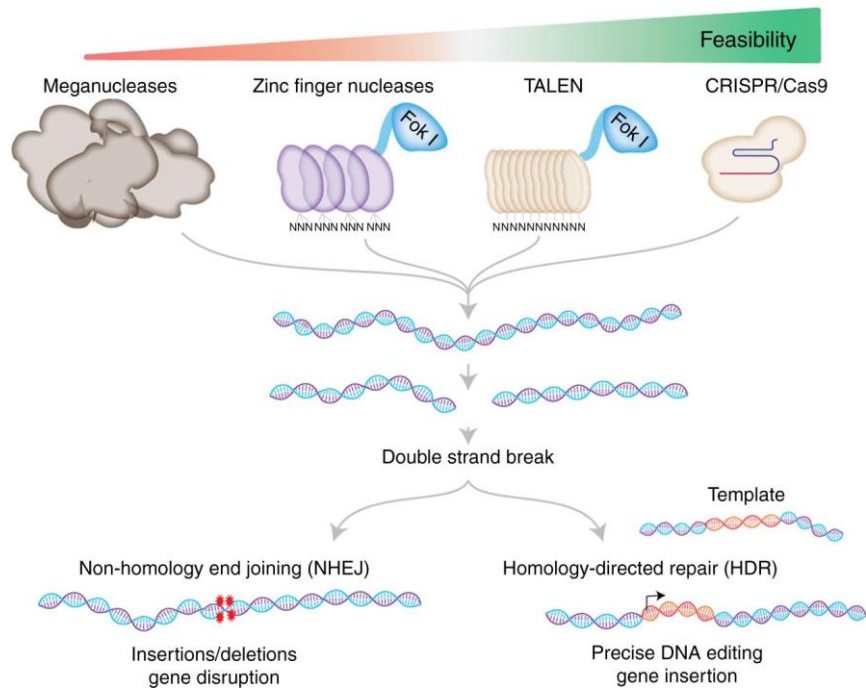

Figure 2

Evolution and different type of gene editing tools. Reproduce from: 2. Adli M. The CRISPR tool kit for genome editing and beyond. Nature Communications. 2018;9(1911).

The rise of CRISPR as a genome-editing technology.

CRISPR stands for clustered regularly interspaced short palindromic repeat DNA sequence, which are marked and separated into the genome by spacers (non-repeating DNA sequence). Genetic sequencing and a subsequent comparison of these genomes allowed to discover that bacteria and archaea had a big presence of CRISPR sequences into their genome and that the spacer sequences belonged to viruses or other mobile genetic elements. In addition, CRISPR components are surrounded by *CRISPR-associated* (*Cas*) genes that encode the Cas endonucleases (Fig. 3).
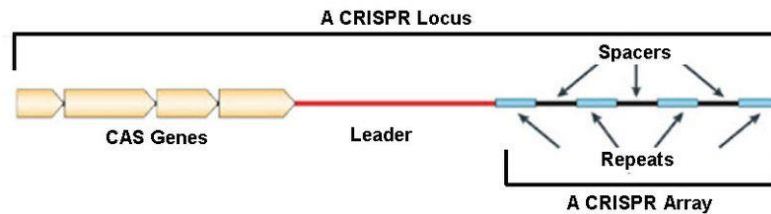
Figure 3

Schematization of the CRISPER complex. Reproduce from: 4. Karimi Z, Ahmadi A, Najafi A, Ranjbar R. Bacterial CRISPR Regions: General Features and their Potential for Epidemiological Molecular Typing Studies [Internet]. Benthamopen.com. 2019.

Following these discoveries, the idea that CRISPR could be a bacterial immune system was born. It was not until the work of Horvath and colleagues that this could be proved. They demonstrated that after a viral challenge Streptococcus Thermophilus bacteria had integrated into its genome new spacers which resembled the virus that causes the infection. Furthermore, based on this discovery posterior researchers showed that the spacer sequences were responsible of the targeting specificity of the Cas enzymes. The short CRISPR RNAs transcribed from the spacer sequences and guided the Cas enzyme to the target site.

When a virus infects a bacterium, it injects its genetic material into the cell. This material integrates into the bacterial DNA and uses its transcription machinery to replicate. Bacterial Cas proteins act like a scan and generate a copy of some parts of the viral material and inserts it into CRISPR sequences, remaining the bacteria immunized for future attacks. In order for the bacteria to be able to recognize the virus in future attacks, the information coded into the DNA must be copied into mobile elements: sequences that act as guides. So, when a virus attacks again this RNA will guide the Cas enzyme to cut and disintegrate that viral material gene (Fig.4)

Another breaking point in the discovery of CRISPR as a genome-editing technology was the identification of protospacer-adjacent motifs (PAMs), a sequence of 2-6 nucleotides that is required for the Cas to function. Siksnys and colleagues [33] showed that CRISPR system from a bacterium was transferable to different bacterial colonies, enabling the posterior biochemical characterization of CRISPR components.

CRISPR became a biotechnology tool when it was demonstrated that Cas9 enzymes can be directly targeted to a desired DNA sequence. This is possible thanks to sort RNAs, the crRNA and the tracrRNA. The first one is composed of a part that acts as guiding sequence (the protospacer) and another part that joins with the tracrRNA. Even though both the crRNA and the tracrRNA are necessary to form the Cas9 protein-RNA complex, Jinek et al. showed that both RNAs could be merged into a chimeric RNA called sgRNA, which had the same functions. This discovery opened up the path to adapt CRISPR for in vivo genome editing in eukaryotic cells.
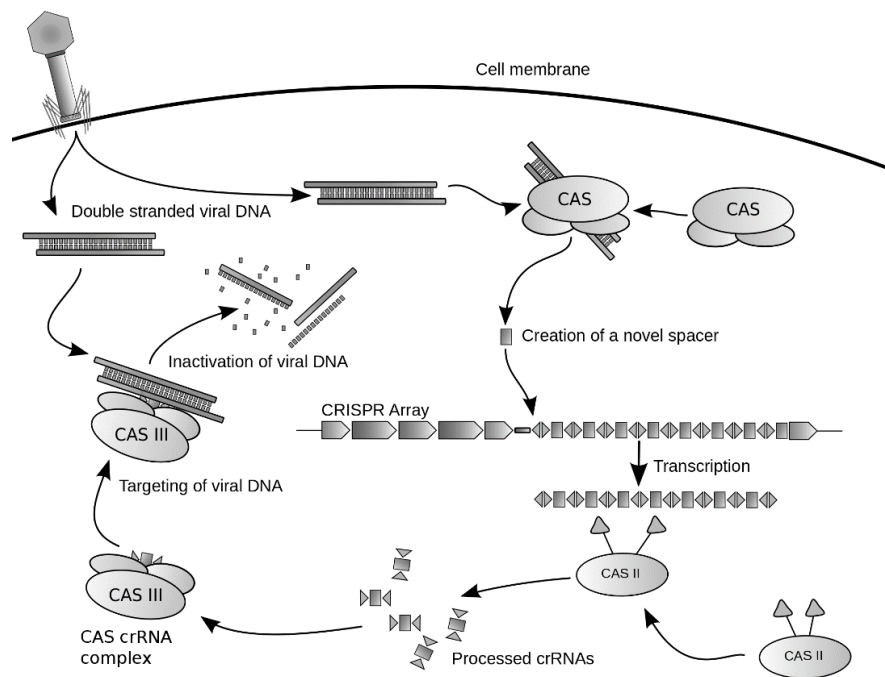
Figure 4

Schematization of the performance of the CRISPER complex as an acquired bacterial immune system against viral attacks. Reproduce from: 5. CRISPR [Internet]. Es.wikipedia.org. 2019.

Types of CRISPR systems.

Mobile genetic elements have been a constant threat to prokaryotes, due to that, new defense mechanisms were born. The different types of CRISPR systems serve to that purpose. The classification of the diverse types was based on the structure of CRISPR-associated (Cas) genes, but with the advance and new discoveries, comparative genomic analyses, structures, and biochemical activities of CRISPR components must also be taken into account. As for genome targeting the most commonly used is the type II CRISPR-Cas9 system from *Streptococcus pyogenes*. It has a simple NGG PAM sequence requirement although it requires two short RNAs. During the last years additional CRISPR/CAS proteins were redesigned for genome editing, as Cpf1 proteins (which only need one sgRNA) from *Acidaminococcus sp* (AsCpf1) and *Lachnospiraceae* bacterium (LbCpf1) (Fig.5).

One important problem that the new Cas9 variants present is their length. Large proteins are difficult to pack and deliver them via Lenti or Adeno Associated viruses (AAV). The problem seems to be overpass thanks to the discovery of new Cas9 variants, as NmCas9, SaCas9, CjCas9. However smaller Cas9 proteins require more complex PAM sequences, bestowing a limited targeting scope and flexibility in genome targeting compared to the largest ones.

Table 1 Naturally occurring major CRISPR-Cas enzymes

	Size	PAM sequence	Size of sgRNA guiding sequence	Cutting site	Reference
spCas9	1368	NGG	20 bp	- 3 bp 5' of PAM	Jinek et al.[42] Gasiunas et al.[43]
FnCas9	1629	NGG	20 bp	- 3 pb 5' of PAM	Hirano et al.[60]
SaCas9	1053	NNGR RT	21 bp	- 3 pb 5' of PAM	Mojica et al.[57]
NmCas9	1082	NNNNG ATT	24 bp	- 3 bp 5' of PAM	Hou et al.[53]
St1Cas9	1121	NNAGA AW	20 bp	- 3 bp 5' of PAM	Gasiunas et al.[43] Cong et al.[45]
St3Cas9	1409	NGGNG	20 bp	- 3 bp 5' of PAM	Gasiunas et al.[43] Cong et al.[45]
CjCas9	984	NNNNACAC	22 bp	- 3 bp 5' of PAM	Kim et al.[56]
AsCPf1	1307	TTTV	24 bp	19/24 bp 3' of PAM	Yamano et al.[50] Kim et al. 2016
LbCpf1	1228	TTTV	24 bp	19/24 bp 3' of PAM	Yamano et al.[50] Kim et al. 2016
Cas13	Multiple orthologs	RNA targeting	28 bp		Abudayyeh et al. 2017

Figure 5

Table of the most commonly used natural endonucleases with the CISPR system. Reproduce from: Adli M. The CRISPR tool kit for genome editing and beyond. Nature Communications. 2018;9(1911).

Re-engineering CAS9

When time comes to reengineering Cas9 proteins there are 3 main points: reduce the size, increase the fidelity and extend the targeting scope. Increasing the fidelity by the alteration of PAM requirement to more relaxed ones has been, until now, the most successful strategy.

One of the most challenging problems that CRISPR-Cas9 system presents are the off-target effects. Researchers have used different techniques as chromatin immunoprecipitation and high throughput sequencing (ChIP-Seq) to map DNA binding sites of catalytically inactive SpCas9 in vivo. The studies show that the regions that are more likely to present of-targets are open chromatin regions, and the distal PAM sites are the places where the system allows more mismatches. Nonetheless, there are a limited number of off-target binding sites in vivo, apparently due to more lose stringent requirements for CAS9 binding instead of DNA cleavage. Even when there are a lot of different studies trying to map all the CRISPR-Cas9-mediated DNA cleavage and binding sites, it is still a challenge. Other studies focus on increasing the specificity of existing spCas9 variants. Some strategies led to the identification of specific point mutations or changing the delivery method from a plasmid-based to a direct delivery as a ribonucleotide protein (RNP) complex. Other approaches include the tandem targeting of a locus with two separated sgDNAs and use the nickase Cas9 (nCas9) or catalytically inactive Cas9 (dCas9) fused to DNA cleavage dominion of Fok I, the control of temporal and spatial activity of the Cas9/sgRNA complex through different techniques or modifying the sgRNA scaffold. Another interesting discovery was that increasing or decreasing the length of the sgRNA guiding sequence was enough to boost up the targeting specificity. Some temporal control over the target activities could be gain incorporating ligand-responsive self-cleaving catalytic RNAs into guide RNA.

A step forward was given with the development of second-generation genome-editing tools. These new tools use nickase Cas9 and are able to precisely change a single base into another without causing DNA DSBs like the first generation. The discovery of this new approach opens the door for CRISPR-STOP editor which alter genetic code by introducing early STOP codons into genes.

CRISPR-mediated gene expression regulation

The discovery of dCas9 was a breakpoint and opened up a huge field of alternative genome manipulation tools. dCas9 cannot cleave to DNA but it can be guided to a specific target sequence, additional to that, the binding activity is so strong that it is able to block the transcriptional process and therefore knock down gene expression. To increase even more the dCas9 gene repression activity, it was fused with the Kruppel-associated Box (KRAB). The KRAB-containing zinc finger proteins form the largest mammal transcriptional repressors and are able to recruit other co-repressor like KRAB-box-associated protein-1 (KAP-1) and an epigenetic reader heterochromatin protein 1 (HP1). It seems that KRAB-mediation produced the loss of Histone 3 lysine 9 trimethylation (H3K9me3) at the promoter gene. The complex dCas9-KRAB not only reduced the chromatin access but it also increased the H3K9me3 at the promoter and distal enhancers.

An induction of gene expression could be achieved by the merge of dCas9 and transcriptional activators like VP64 (4 tandem copies of VP14 of VHH). Furthermore, to make the transcriptional activators even stronger, second generation dCas9 platform is fused with a transcriptional complex conformed of VP64, P65 and Rta (VPR) proteins. To reach the activation of endogen locus, instead of directly fusing to dCas9, the effector domains of the transcriptional complex could be recruited at the targeting site by sgRNA scaffold. MCP, SAM and Suntag, some examples of sgRNA scaffold approaches, prove to be more efficient than the traditional VP64 approach.

CRISPR-mediated epigenome editing

An important number of diseases including cancer present aberrant DNA methylation. On this ground, researchers are focusing on developing locus-specific epigenetic editing tolls that specifically target aberrantly regulated loci, with therapeutic potential. To achieve that goal investigators utilized dCas9 system to deposit DNA methylation marks and to remove the endogenous DNA methylation from a specific loci.

To deposit DNA methylation marks, dCas9 is fused with catalytic domain of DNMT3A or MQ3. In addition, the target recruitment of repressive epigenetic machinery increases the robustness of DNA methylation sustained the gene repression for much longer. However, some studies highlight that this method has off-target effects like leaving

methylation footprints which are independent of method delivery or sgRNA, but surprisingly with a limited impact on gene expression.

Endogenous DNA demethylation, in vivo cells, is mediated by ten-eleven translocation (TET) proteins. In order to achieve locus specific DNA demethylation through CRISPR technology, researchers use dCas9 as a platform to recruiter TET proteins, more specifically TET1. Although it seems to present an important increase in mRNA after the specific demethylation it is not known what off-targets effects it could present.

Another important stilt on epigenetic manipulation goes through understanding the mechanism behind histone proteins regulation and the downstream effects that produce. To achieve that, researchers are exploring the possibilities of using dCas9 as a platform to recruit histone modifiers to a specific locus. Until now investigation was focus onto locus specific methylation, acetylation or removing such marks. Remains uncertain if this approach generate the same off-target effects than the others chromatin editor. A promising study is based on dCas9-LSD1 fusion complex which is used to manipulate the regulatory activity of distal enhancer regions though removing H3K4me2 mark.

 By contrast another way to epigenetically manipulating enhancer function is to locally deposit H3K27 marks. A fusion between dCas9 platform and acetyltransferase P300 translates into an increase in local H3K2ac levels at enhancer regions. The complex resulted from the merge of dCas9 and PRDM9 is able to modify the activity of H3Kame3, inductor of reexpresión of silenced genes. Another approaches are to use instead dCAs9-HDAC3 complex.

The above-mentioned epigenetic manipulation tools are based on overexpression of the complex formed by dCas9 and epigenetic modifiers. Nonetheless this overexpression they could leave a low but global epigenetic footprint into the genome. Some new strategy is based on using endogenous epigenetic chromatin regulators (FIRE-Cas9) to increase the local target specificity.

With this tools on hand researchers have used them to realize high-throughput screenings to characterize functional distal enhancers, targeted reprogramming of lineage specification, generation of induced pluripotent stem cells and reversal of HIV latency.

The causal relationship between the epigenetic mark and its regulatory impact remains unclear and suppose a real challenge. In addition, with the use of dCAs9 and epigenetic modifier complex it is not sure if the regulatory activity is due to the complex or the epigenetic marks. Into this loose ends are focused the future research.

CRISPR-mediated live cell chromatin imaging and manipulation of chromatin topology

Tools based in dCAS9 platform supposed a huge advance into the genome targeting for live cell chromatin imaging. To achieve this, dCas9 has been fused with fluorescent proteins that acts as fluorescently labeled. This complex is able to mark specific repetitive sequences. Marc Non-Repetitive sequence is trickier because of free-floating fluorescently labeled dCas9 proteins. The problem it seems to be solve by the using of engineered sgRNA scaffolds which contains up to 16 MS2 binding modules to creates a strong fluorescent signal amplification and allow imaging a repeat genomic region with as few as 4 sgRNAs. This technic allows as to track native chromatin loci throughout the cell cycle and determining differential positioning of transcriptionally active and inactive regions in the 3D nuclear space.

To understand better the function and contribution to gene expression of the chromatin structure, researches had engineered an artificial chromatin loop. With the help of this tool it could be possible to form new enhancer–promoter connections to overcome certain genetic deficiencies beside aberrantly active enhancer–promoter interaction can be inhibited. Studies as Morgan et al had proof the power of CRISPR as a targeted chromatin structure-rewiring too.

Large-scale genetic and epigenetic CRISPR screening.

CRISPR KO screenings is a tool for large-scale functional genes screenings. The mechanism behind this tool is to deliver a large pool of Cas9/sgRNAs, with6 –10 different sgRNAs per specific gene, into a population of cells via a low multiplicity of viral infection. If a gene is essential for a phenotype to express the cells that presented it will be mark by sgRNAs and with time will depleted from the population. Given that each sgRNA is fully integrated into the genome of those cells, the sequence guide for each one could be used as a barcode. Even more, the amount of sgRNA in certain cells could be quantified by targeted sequencing.

Future directions.

It cannot be denied that CRISPR-Cas9 has been one of the biggest discoveries of this century. Not only it supposes a trampoline to molecular biology research but also has revolutionized other fields like medicine, pharmacy, genetic or agronomy. Even though the perspectives that this technology opens are very wiled, it's important not to forget that to achieve the full potential of CRISPR-Cas9 technology into therapeutic applications some problems must still be solved. First, the size of existing Cas9 proteins is a challenge to pack them into the AAV vectors. The problems could be solved with the discovery of smaller Cas9 proteins or finding a way to reduce the size of existing ones.

Another aspect that must be taken into account when applying CRISPR-Cas9 technology to the clinic is the potential immunogenicity of Cas9 proteins. Humans have already been in contact with the bacteria from where Cas9 proteins are obtained, so there are studies that hint on a pre-form immune response to them. To solve this issue, it is necessary to find orthogonal CRISPR-Cas9 proteins to which humans have not been introduced before.

Finally, a technological advance such as CRISPR entails new and complex ethical problems. The CRISPR-Cas9 technology is able to take a specific gene, with advantageous feature, and spread it to a whole population even to a whole species. This phenomenon is called "gene drive" and is a long-term outcome. Before implementing such an advantageous feature, it would be important to have a deep consideration of its possible consequences.

Objective.

The main goal of this work is to learn about the CRISPR/Cas9 technology, how to use it and try to theorize about its possible application for genetic edition of early embryos to specifically correct the Huntington Disease.

I chose the Huntington disease because it has no current treatment and presents a complicated diagnosis that encompasses an interdisciplinary management, not to mention that it implies a rather stressing diagnosis for the patient and his offspring. By using CRISPR/Cas in zygotes I pretend, at least in theory, not only to prevent the onset of the disease, but also to eliminate its transmission and genetic anticipation.

Materials and methods

<u>Methods</u>

Beverly L. Davidson [1] and her group have been working with the idea of treating HD using genetic editing for the last ten years and with the emergence of CRISPR/Cas9 they have focus on it. I choose their experimental work as a guide for this work.

As described in the Introduction, the HD is an autosomal dominant disease, with a common presentation in heterozygosis, and very rarely in homozygosis. Taking into account this fact, I will focus on heterozygosis, living out those cases that have a homozygosis presentation. Moreover, it is important to mention that the penetrance of the alleles depends on the number of repeats. The bigger the number of the repeats, the higher the penetrance.

The mutant allele is characterized by ≥ 36 CAG repeats in exon 1 of the *Huntingtin (HTT) gene*. The presence of intact PAMs motifs is crucial to an important cleavage efficiency. The PAMs motifs vary according to prevalent Single Nucleotide Polymorphism (SNPs). Since the *HTT* regulatory region spreads within the first 5' two kilobases, it is quite easy to use SNP-dependent PAMs as a target for CRISPR/Cas 9. This fact is important to understand how CRISPR/Cas 9 can be directed to act only in the mutant allele and not on both alleles.

To design the gRNA specific for the mutant allele, firstly the genome has to be scanned to identify the most prevalent SNPs (Fig. 6) in the critical area that could act as a target. In order to edit the *HTT* allele responsible of the disease in a heterozygous situation, it is necessary to search the flanking regions of exon 1 to identify a SNP that lead to the gain of a PAM sequence in the mutant allele or loss of PAM in the normal allele. Once we have chosen the SNPs that may suits the experiment purposes we can design the 20 nt-guiding sequence for the sgRNA that will be complementary to those SNPs.
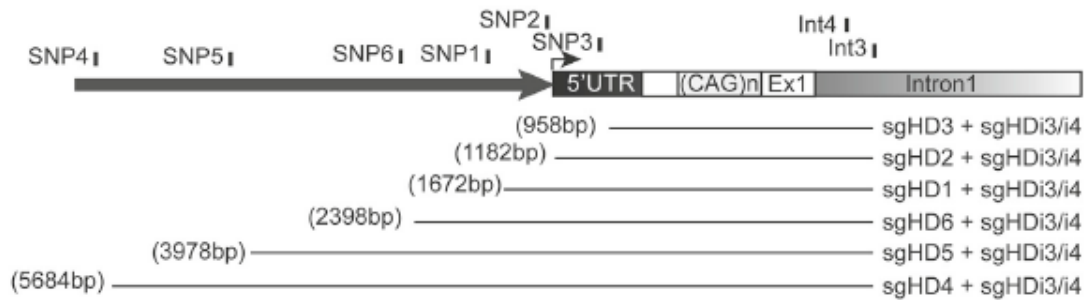
Figure 6. The image shows the 6 most prevalent SNP that at the same time allows to loss or gain PAMs. Reproduce from: Johnson C, Davidson B. Huntington's disease: progress toward effective disease-modifying treatments and a cure. Human Molecular Genetics. 2010;19(R1): R98-R102.

Once we have that sequences and the sgRNA are designed, it can be fused with SpCAS9 system from *Streptococcus pyogenes* and with-it ensemble the CRISPR/Cas9 complex with the specific target (Fig. 7)
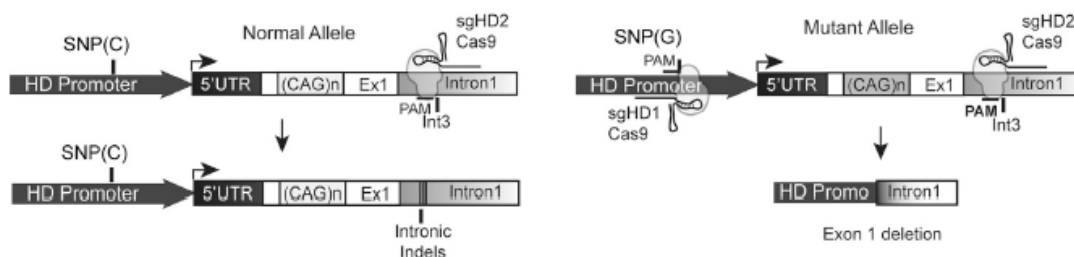

Figure 7. Schematization of the functioning of CRISPR / Cas9 on the mutated allele. Reproduce from: Johnson C, Davidson B. Huntington's disease: progress toward effective disease-modifying treatments and a cure. Human Molecular Genetics. 2010;19(R1): R98-R102.

Materials

To identify the SNPs that CRISPR/CAS9 will use as a target it is needed to screen the *HHT* specially the exon 1. To do this, the NCBI website and the 1000 Genomes database can be used.
NCIB web: https://www.ncbi.nlm.nih.gov/pubmed/

In this particular case the SNP of interest will determine the gRNA to be use. In order to predict the possible off-target of the selected gRNA the Brealing-Cas, an online tool for gRNA design, is used. To do so, a 500bp genomic sequence corresponding to the area targeted by the gRNA is obtained from the UCSC browser and transformed to FASTA format to be use as input for the Breaking-Cas program. Breaking-Cas will provide and efficiency score based on the number of possible off-targets for the desired gRNA. The score retrieved from the Breaking-Cas will determine the suitability of the selected gRNA, were the higher the score corresponds to a lower number of possible off-targets.

Breaking-Cas web: http://bioinfogp.cnb.csic.es/tools/breakingcas/

Results and Discussion

It is impossible to deny that CRISPR has supposed a big revolution in the field of bioenergy and it is only the beginning because there is still much to discover yet. What started being a bacterial acquired immune system has turn up to be the most important tool of this century. At present the fields in which CRISPR has outstanding applications are medicine, pharmacology and agriculture.

In the field of medicine CRISPR has supposed a step forward in the treatment of genetic diseases that were impossible to treat before by the standards tools. CRISPR provides an easy to use, economic and quick tool that allows to treat these diseases in a safe way or at least, that is what suggest the studies so far carried out. Al list for the moment the main application of this new tool is in the cases of the monogenetic diseases in somatic cells. It is important to have this in mind because there are some differences on working into somatic or geminal cell lines. Firstly, somatic cells usually are differentiated cells while germinal cells are not, and secondly and most important the changes that could be made into the somatic line of cells only affects the individual of whom belong, meanwhile the changes that are made into the germinal cell line can be pass to the descendants.

Not only the genic treatment area has experienced an advance due to CRISPR/Cas technology, the area of diagnosis has experienced it to. Beside the genetic sequencing and this new tool, a specific diagnosis is easier and more precise to make. An example of this is the use of CRISPR into live cell chromatin imaging.

In the field of pharmacology CRISPR has a much bigger impact. With the discovery of this tool the idea of a personalized treatment is even near to become a reality. Not only it will allow to design drugs that are target specifically what is even more those drugs will be specific to person that were designed for. As an example, could be the use of CRISPR for determine the genetic profile form hereditary cancer and try to adapt the treatment in function of if the patient presents or not that mutations. This simple fac will help when came time to decide which treatment will work better for the genotype of the patient.

Onto the fields of cattle raising and agriculture the discovery of this tool has supposed something resembling of the discovery of Holy Grail. Basically, CRISPR allows to edit de genome and select those genes that will generate the best cattle or plants, one which will have the most desirable features and remove those that doesn´t interest in that moment. Furthermore, it is possible to create transgenic plants that are resistant to any type of plagues or transgenic products that are able to grow in any type of soil not needing to take into account the specific climatic needs. In the case of the cattle raising, it could be created specific animal breeds based on the needs or the wishes of the company that requests it. Moreover, it is possible to combine different features from different species.

One of the main point of using CRISPR/Cas into the cattle raising is for the feedback that offers the line of investigation on embryos. In human embryos, at least at the moment, it is illegal to apply a new technology that it isn't totally confirmed his sides effects, but

the same rule doesn't apply to its use in animals. Therefore, the experience the information that can be obtained by experimenting on animals is so important. Having more information, especially about the side effects, will support the jump to humans.

<u>Advantages</u>

As mentioned above, CRISPR/Cas has been the discovery of this century, and not only for its applications but also because its characteristics:

Effectiveness: CRISPR/Cas has proven to be more effective than the previous gene editors. This may be due to the fact that CRISPR can use homologous repair which increases targeting efficiency and partially avoids the error-prone non-homologous end joining DNA repair mechanism which could be the cause of new error or DNA alteration at the breaking point.

Specificity: CRISPR/Cas is composed by two complex one of them being a sgRNA that has a complementary sequence of approximately 20 nt that is complementary with the region of the genome we want to target. Having such a precise way to target a concrete region considerably increases the specificity resulting in less off-target effects than the previous gene editors.

Easy to use: Since nowadays here are a lot of databases of gens it is quite easy to delimit the region where CRISPR is going to act and based on that create a specific sgRNA. Not to mention the fact that there are a lot of websites which are specific to create these guide sequences, even more, there are already database of specifics sgRNA.

Since the whole complex of CISPR/CAS is not bigger than the previous gens editor it can used the same excipients or even some smaller ones, to deliver it.

Cheap: Since that over the years the cost of genetic sequencing has decreased and the appearance of databases that are free the cost to realize the sgRNA is quite affordable. The major part of the cost of CRISPR goes to obtain the CAS9 complex and the excipient in whom is gone be delivered into the cell. Another part of the cost consists of the controls that evaluate the CRISPR activity. Overall, the price does not exceed a few hundred euros or at most a few thousand.

Quick: Once the CRISPR components have been delivered, to see the results it will only take about a few hours. It depends on the time that the transcription machinery will need to read the alterations that have been made on the site that have been targeted. Sometimes, especially in those cases that it is important to observe if there is an increase or decrees of the production of a specific protein it could take more time.

Several simultaneous changes: As it has been mentioned before CRISPR it is not only a genetic scissors. Now a day several changes could be made at the same time, like cutting a specific sequence and replace it with a donor of our choice. This supposed a breakthrough, since it allows to make several changes without the need of repeating the procedure and without increasing costs.

Advantages of using CRISPR/CAS9 to gene-editing embryos to treat Huntington Disease:

Huntington Disease is the paradigm of a disease with an expansion of a three-nucleotide repetition as pathogenic base. Given that it is a monogenetic disease with a well localized gen, it is potentially a model disease in which the technology CRISPR/CAS can be applied. Currently, the lines of investigation focus on the possibility of applying this tool in adults and differentiated cells to treat the disease, with promising results.

Here we take a step forward and try to apply it to correct it in embryos. One of the biggest concerns about this type of disease is the genetic anticipation. Now a day the only method to prevent this is the genetic counseling or in the case of those women who are already pregnant, abortion is allowed.

Because of the autosomal dominant inheritance pattern of HD, the selection of unaffected zygotes should be the preferred method. However, based on my personal interest in HD, I selected this disease to learn about the possibilities of the application of the CRISPR/Cas to correct genetic diseases.

Correcting the genetic alteration with CRISPR/CAS in the embryo, prevents the disease and also its transmission to the offspring. Other interesting effect of treating the disease in an embryonic cell is that the mutant protein will not be deposited in the cells and therefore will not have its toxic effect allowing a correct neurodevelopment, reducing the odds to suffer other diseases that are associated whit the pathogeny of Huntington.

Disadvantages

Like others greatest discoveries CRISPR/Cas system has his floods and it is equally important to be aware of them as the knowledge of his advantages. Going through understanding these floods is how this technology will still evolve in the future and we will be able to use it in a safer way and extended to more fields.

Off-target effects: This has been the biggest concern since the CRISPR/Cas technology started to be used. Even when CRISPR is so target specific it seems to have some off-target effects. This is due to the fact that the 20 nucleotide sequence of the gRNA that targets the specific region of the genome allows for mismatches and therefore anneal at a non-desired genomic location producing non desired side effects.

Its mechanism of action is still not fully understood: Part of the difficulty of limiting the side effect resides in the fact that a big part of the genome and his mechanisms of regulation are still a mystery. It is complicated to try to use a gene edition tool without knowing a hundred per cent how the genome is organized, the way it is regulated and the external elements that influence its regularization. Furthermore, the CRISPR/Cas technology is relatively recent and the knowledge that we have about it is that one has been presented in bacteria or in vitro models, there is not much knowledge about its behavior in the human body and it is difficult to ensure that it will behave in the same way.

Long-term effects: Since this tool is brand new is quite difficult to predict what long-term effect its use will have. Without any kind of feedback, it is impossible to predict how the genetic edition will affect the human body and if the are some new and unknow side effects. What is even more important, is the fact that we are unprepared for facing those effects.

Lack of standardized control: Being such a specific tool does not allow for a standardized method of control. In fact, it is needed a specific and appropriate control for each situation. Initially this may seem like a good think but having such a restrained vision it is possible to overlook secondary effects that are more general and not associated them with the use of the tool. Another problem of not having a standardized control is the fact that some researchers can take advantage of it and falsify positivized some of the results of their research.

Lack of detailed studies: In the same line as the previous problem, the fact that is a brand-new discovery has not given time to carry out more detailed studies.

Lack of patients: The use of CRISPR in humans is still under a big debated. The experiments that have been done so far show a very promising perspective but a large part of them have been made in vitro or in animal model and not onto humans. Not to mention that the unregulated experimentation is forbidden in humans.

The use of CRISPR/Cas9 for gene editing in embryos.

This possible use of the CRISPR/Cas technology has risen a lot of different and contradictories opinions. On one hand there are those who defend the use of CRISPR on germinal lines arguing that the benefits of it could help to improve the human species and the environment. On the other hand, are those who are opposed to such practices either because of etiologic ideas or because there isn't enough guaranty for a safety use or not insurance for a no perverse use.

Despite of the controversy it is undeniable that CRISPR has the potential to allow us to modify the embryonic genome.

Importance of CRISPRs and the new horizons it can open

Since the discovery of CRISPR and its role as an acquired immunity system which is pass by through generation of bacteria's, it occurred to researchers the possibility to use CRISPR in the same way or at least to use it for enhance those genetic traits that are more desirables and eliminate those that courses with a disease.

The ability of manipulate the genome of a germinal cell line allows as not only to prevent the disease from developing, but to eradicated if for the future generations. There is always going to be novo mutations but at least in those cases on which the mutation is pass by we can stop it. Some genetic diseases could even be eradicated.

The other great pillar that substances this type of application is the fact that by manipulating the genome of a germinal cell line it is possible to insert new genes or eliminate them in order to create an immunity against certain diseases. On the other

hand, it can have a more frivolous application as is the ability of chosen those features that we want for our descendants.

Uncertainty about the procedure

Even when CRISPR is so target specific it is important not to forgot that this specificity depends, or at least part of it, on the type and the grade of differentiation of the line cell. An 8 cells embryo is pretty much a cluster of undifferentiated cells and it is difficult to predict how this fact could affect the capacity of CRISPR to target and join the right place and not a random one.

But before considering those problems it is important to understand how those embryos are obtain, when is the moment of acting on them or the moment for running control tests in order to see if the experiment has succeeded.

In order to work onto the embryonic genome, it is necessary to obtain them first. To do this, we go through in vitro fertilization.

One of the first steps in this procedure is the use of short ovarian stimulation protocol by using gonadotropin-releasing hormone (GnRH) antagonists as a stimulator.

GnRH antagonists belong to the family of GnRH analogs and are used to control ovulation. They avoid the hormonal peak of LH and in this way, they prevent ovulation from occurring spontaneously at an unwanted time. It is important to know exactly when ovulation occurs in order to perform the ovarian puncture at the right time. By slowing the LH peak these drugs also have an effect on the endometrial receptivity. This is especially useful in those cases in which it is proposed to make frozen embryo transfer or ovodonation, because it will help to prevent an early rejection. These drugs are also used in the treatment of endometriosis or in the case of hormone-dependent tumors.

GnRH antagonists act as a competitive antagonist that bind and block the GnRH receptor located in the gonadotropic cell membrane of the pituitary gland. The blockade produces an inhibition of the synthesis of the gonadotrophins (FSH and LH) involved in the regulation of the menstrual cycle, and therefore slowing the ovarian activity.

One of the advantages of these drugs over other analogs that have been used previously, is the production of an immediate suppression of serum levels of LH preventing spontaneous ovulations. Since the effect occurs in a few hours it can be administered at any time that there is a threat of LH peak which could not be done with the agonists ones since they needed between 7 - 10 days to reach their effect.

Thanks to this rapidity of action, the duration of the treatment can be minor (10-12 days instead of one month). This translates into practice as fewer injections less and less face-to-face consultations during the period of ovarian stimulation. Since it is a less aggressive stimulation, minor doses of FSH can be administered and therefore there are fewer side effects and they are better tolerated. By needing a smaller dose of hormone, the cost of treatment is also reduced.

This type of therapy allows the concomitant use of GnRH agonists instead of hCG to induce ovulation reducing whit it the risk of producing ovarian hyperstimulation

syndrome (OHS). Presenting the advantage that it can be use in young women, donors or those women who present polycystic ovary syndrome where the risk of OHS is higher.

Being a less aggressive treatment in general lines, it makes it a very well tolerated treatment.

One of the drawbacks that presents is the fact that the pregnancy rate achieved by this protocol compared to those that use GnRH analogues, is slightly minor (3% -5%). This is due to its effect on the endometrial receptivity. Before starting the treatment, oral contraceptives or estrogens must be administered in order to avoid asynchrony in the follicular growth that could negatively affect the success rate of ovule retrieval.

In order to achieve ovarian stimulation, it is not enough only with the GnRH antagonist regimen, it is need to administrated gonadotropins first to produce the follicular growth and then administrated GnRH agonists in order to achieve the induction of ovulation.

The fact that most drugs are injectable offers the big advantage than facilitates the self-application by patients being no needed face-to-face consultation.

The control of ovarian stimulation is carried out through the control of blood levels of gonadotropins and a vaginal ultrasound that allow to see the follicles. When the desired number and size are reached, is the perfect moment to administrate the drugs that will induce the maturation. Once we have the mature ovules, we proceed to extract them. The procedure is done by ovarian puncture and aspiration of the follicles being all the process guided by a transvaginal ultrasound. The procedure is performed under anesthesia and requires that the patient remain under observation.

Despite all the steps that has been followed the quality and viability of the ovules it cannot be predicted until they have obtained and classified them. Those ovules which are mature and with normal morphology, previously selected spermatozoa, is introduced by intracytoplasmic microinjection. The following day the number of fertilized ovules is determined, those ones are kept in the laboratory for 2 to 6 days until the more vials are implanted.

Three days after the post-puncture, is going to be done an extraction of the polar corpuscle or one / two cells from the pre-embryo. The sample is subjected to a PCR to see if there is amplification of the mutated allele. Those ovules whose samples did not present amplification of the genetic material at 4- or 6-days post-puncture are transferred to the uterine cavity through the vagina. This procedure is called embryo transfer.

Only 3 embryos can be transferred in each cycle to increase the chances of pregnancy. Those pre-embryos that are not transferred are frozen (Fig. 8)

Usually those frozen pre-embryos are the origin of most of the embryos that are used in research. It is important to mention the fact that these embryos do not have a reproductive goal and only serve to fulfill the purpose of a in vivo model.

It is therefore not surprising that rise of doubts about the procedure. On one hand we must bear in mind that the samples are difficult to obtain, and it is not an inexhaustible source, not to mention the legal needs that involves its acquisition. And on the other hand, in spite of having these samples, it cannot be assured that the CRISPR machinery will acts as we wish.

Figure 8. Schematization of the process of obtaining embryos by in vitro fertilization. Reproduce from: 8. http://jcgaal.com J. Ingenes | Líderes en Reproducción Asistida de Latinoamérica [Internet].

Uncertainty about the results

One of the biggest problems about genetic experimentation on embryos is the fact that long-term side effects onto the individual and his descendants are unable to be predicted.

Firstly, there are the side effect that could be caused by CIRSPR technology. As it has been mentioned before Crisper is a very efficient and specific tool, but it is not exempt of having failures. Probably the most concerning one is the presence of off-target effects and the mutation that could provoke. The programs that designed the sgRNA usually could give an approximation of the percentage of the off-targets that are expected. But it will not be until the first controls when it is possible to confirm the presence of those off-target and to theorized about the possible effects that may have. Being not able to predict the off- targets effects makes it difficult to be prepared to face or treat the sequels that can produce.

Secondly, there are the side effect that are caused by the response of the body to the elimination or gain of a gen. Every gen has his own porpoise, but it also takes part in a bigger machinery where is only a piece. When we eliminate or introduce a gene not only it is altered the individual function but also is alter, he whole machinery. Now a day it is still difficult to affirm with certainty how a genetic change will affect the body and whether it will be presented in the way it is expected or not.

It should be noted that in the case of Huntington's disease this is even more difficult since *Huntingtin* is a ubiquitous protein that is involved in a manner still unknown in neurodevelopment. Eliminating the mutant allele theoretically prevents the deposit of mutated protein, but it is not known for sure how the new resulting protein can act at other levels of the brain and if it will have the same quality and function that the normal one.

Ethical, legislative and regulatory problems involved in the genetic edition of embryos.

The ability to edit the genome is quite new because until now we do not have the tool or knowledge to do it. During this century new discoveries have been made and along with that much progress was made in the field of biological engineering to the point of discovering tools that allowed us to change the human genome. Along whit the developing of new tools for genetic editing, concerns about the way this tool may be used rise to. Within these concerns are those of those who are not agree whit the idea of changing the genome that has been given to us, either due to religious beliefs or personal beliefs. By contrast, there are those who believe that since we have the power to control our own evolution is our duty to achieve a better human condition as long as that does not lead to the domination of a group over the rest. Some way in the middle are those who believe that the advance of science is important as long as it is carefully regulated and only for therapeutic purpose.

The ethical debate about the problem of genetic editing in germinal human lines it has been going on from decades. In the 70 with the discovery of the recombined DNA there was a great wave of concern about the possible to use this discovery as a biological weapon and the inability to stop it in case it was disseminated. As a response to the increasing uncertainty and unrest within the scientific sphere, a moratorium appears in which are established the conditions in which this type of research has to be carried out. The moratorium reflects the agreements achieved at the conference of Asilomar, in which was committed to a self-regulation of the scientific work in which the investigations were carried out with transparency and public participation in the scientific policy. During the decade of the 80s the prudent ideology was replaced by the profit motive, being defined by the expression of Laissez faire in Wall Street. Today it is preferred a medium position: we must be aware of the risks of eugenics and the insecurity of the practice, due to the lack of knowledge or perverse use, and therefore we must set a series of directives for the performance.

While everyone agrees onto the fact that CRISPR/Cas9 is a very useful tool with a big potential, not everyone is on board with all the application that could have. Until now the research into somatic cell lines has not been a problem beyond the general request to be done safely and with transparency. The problems appear when it comes to experimenting on geminal lines.

In 2015, rumors about the possible application of CRISPR technology onto germinal cell lines by various Chinese research groups began. The reaction of the scientific community was prompt. The major scientific journals published almost simultaneously manifestos signed by several scientists who highlighted the need for dialogue priority on ethical boundaries when using CRISPR in human embryos. The main reasons for rejecting this

kind of research are the fact that the technique is not yet in a position to be applied with security and neither is it possible to predict the effects on future generations, the fact that there are alternatives such as preimplantation genetic diagnosis or prenatal diagnosis, risk of using the technology for non-therapeutic purposes and the loss of trust on the part of the citizens if reckless experiments are carried out. The authors of the manifesto, including J. Doudna and G. Chuch propose the implementation of a moratorium and the promotion of the dialogue. They also propose temporarily postpone the genetic editing in embryos until the technique is more secure and then discuss its application. This will also give citizens time to get used to the new discovery and its possible applications without losing their trust.

In 2015 the *National Academies* of United States together with the *Royal Society* of the United Kingdom and *Chinese Academy of Sciences* held an international symposium monographically dedicated to the ethical aspects establishing:

- Maintain research in pre-cyclic stages and in those case in which are use human embryos or germinal cells, those will not be used for reproductive purposes.
- Support research in somatic cells lines since it provides important feedback, as long as it is done in the regulatory framework of gene therapy
- Reject momentarily the genetic editing in human germinal cell lines until a certain degree of safety has been reached in the use of the technique
- Creation of a permanent debate forum

The foundations that were behind all these proposals are: The applications of the genetic editing offer an extraordinary improvement onto the quality of life and the environment, but it can be dangerous if it is not used with prudence or for non-therapeutic purposes. The social alarm leads to restrictive measures that prevent the advancement of science; therefore, it is important to carry out transparent practices and educate and inform citizens about the way of functioning and the purposes of the new genetic technologies. Embryos in early stages are not an end in themselves and therefore they can be used for research as long as they are not implanted later to achieve a pregnancy.

Not only the scientific community has been included in this debate, the need for an updated legal regulation was been required.

The European Union prohibits the practices of eugenics, the practices that are aimed the selection of specific groups of people and the cloning of human beings. Surprisingly in the regulations there is no reference to gene editing. In 2016, with the increasingly expanded use of CRISPR, the Group on Ethics in Science and New Technology provided a brief statement on the regulations that should be followed in these cases.

The Council of Europe has been the pioneer institution in terms of regularization in matters related to the genetic industry. This institution allows the experimentation in human geminal cells as long as it is for therapeutic purposes and human rights are respected. The Council promote the Oviedo Convention as a reference for international debates which involves the area of genetic editing, since it is the only legally binding international instrument dedicated to the recognition of human rights in the field of biomedicine.

It is important that science advances go hand in hand with a solid legislative framework. The recent events related to the use of the CRISPR have shown that it is needed to have the same legislation, at least in terms of genetic therapies, for all the countries, avoiding in this way the use of countries with lax legislation as laboratories for hasty experiments or those who violate the human rights. When the techniques are so brand new it is favorable to establish some fundamental principles and build a dialogue upon them, instead of a strict legislation.

Conclusions

CRISPR as a tool

CRISPR has been the discovery of this century and it has supposed a true revolution in the field of bio-engineering. It was discovered in bacteria where it is considered an immunity acquired system.

It is a solid tool that presents very specific characteristics. It is composed of two subunits, an RNA sequence of about 20 nt which is complementary to the target DNA. This sequence serves as a guide for the subunit Cas9. Cas 9 is an endonuclease which will subsequently make the cuts at the indicated site. The CRISPR/Cas system is known as a genetic scissor. In recent years it has been shown that this Cas9 unit can be replaced by other endonucleases that presents the same cutting function as the Cas9 or on the contrary it can have other functions. The discovery of those new functions and their applications allowed new applications for the CRISPR system. For example, the marking of the chromatin with CRISPR has allowed to understand better its composition and the mechanisms that interfere in its regularization.

Another important feature of the CRISPR/Cas system is the fact that its use is cheap and quick. Thanks to the appearance of online databases it is very easy to design the sgARN in the computer without any cost.

Like other tools the CRISPR / Cas system presents its limitations. The most important one is the presence of off-target effects. Some of them can be predicted during the design of the sgRNA, but until we do not perform the subsequent controls, we will not be able to verify the quantity or the location of these off-targets. Another problem that this technology presents is the fact that we do not know the long-term effects that may arise from the mutations caused by the off-targets.

This tool has revolutionized a lot of fields not only those that are related to health. Its applications are very varied, ranging from changes in the human genome to changes in the environment. Despite all this, there is still potential to be discovered.

The use of CISPR/Cas for genetic editing of human embryos

The editing of human geminal cells has always been a controversial and complicated goal to reach but with the arrival of CRISPR technology, it can be at the reach of the hand.

Giving the high efficiency of CRISPR it is seems to be pretty easy to edit human embryos just like any other type of somatic cells. But it is really that simple? No, it is not. There are some important obstacles that we must overcame in order to achieve this goal.

Firstly, the access to human embryos is very limited. Usually the samples came for fertility clinics or donor that have frozen their pre-embryo from previous attempts of in vitro fertilization. In order to obtain those pre-embryos, first it is needed to obtain the ovules. To obtain the ovules it is necessary to subject a woman to an ovarian stimulation process. It is a process with many critical steps and quite expensive, which does not guarantee the obtaining of viable elements.

Secondly, CIRSPR/Cas it is not an infallible technology. It has its own failures that have been mentioned above. It is important to acknowledge the fact that the specificity and the efficiency of the tool may vary depending on the type of cell and its range of differentiation. It is possible that the off-target effects that usually CRISPR presents could be intensified in embryos since they are immature and poorly differentiated. But the biggest problem remains to be the inability to predict how these off-targets will affect the resultant individual and the offspring.

Third, he legal framework is very complicated. It is a practice that is prohibited in many countries or is only allowed under very strict permits with very clear purposes. Usually these purposes are related to therapeutic purposes and the studies have to remain on the pre-clinical phase. Giving that it is quite difficult to extrapolate the results obtained to the individuals that will result from the embryo.

It is therefore a complicated procedure that for now is better to leave on pause. With time, the side effects of the CRISPR will be better known and it will also give time for the legal framework to adapt to this new technique.

The use of CRISPR/CAS in the editing of embryonic genes for Huntington's disease.

Huntington's disease is a hereditary (AD) neurodegenerative disease that is characterized by presenting in the exon 1 an amplification of the triplet CAG beyond 36 repetitions. The protein resulting from the mutated allele is deposited in the neurons causing neurotoxicity with gradual deterioration and atrophy of the caudate nucleus and striatum. It presents a combined clinic of motor deterioration, cognitive and affective deterioration. It has a late diagnosis due to the joint involvement of many areas and presents genetic anticipation. It does not have an effective treatment. Now a day to prevent the genetic anticipation the only options are the preimplantation genetic diagnosis or the prenatal genetic diagnosis, living as an option, in the case of the last one, the abortion. I have chosen this disease for this work due to the genetic anticipation and the fact that Huntingtin plays an important role in neurodevelopment.

Because of the autosomal dominant inheritance pattern of HD, the selection of unaffected zygotes should be the preferred method. However, based on my personal interest in HD, I selected this disease to learn about the possibilities of the application of the CRISPR/Cas to correct genetic diseases.

So far, experiments with CRISPR that have been performed in somatic cells have shown a remarkable reduction in the level of mutated protein, but it cannot be said that the disease has been treated. Furthermore, the problem that supposes the genetic anticipation still remains there. Thus, the mutated protein has already generated an abnormal neurodevelopment that cannot be reversed despite its elimination in the adult live.

The use of genetic editing in embryos by CRISPR / Cas could eliminate the mutant allele presenting from the beginning of the life a healthy protein that does not accumulate in the cells and allows a normal neurodevelopment. In addition, by eliminating the allele where the expansion of the triplet is present, genetic anticipation is prevented. Since we are working in the zygote, the changes that we achieve will pass to the offspring.

Theoretically it sounds possible but in reality, this assumption is more difficult not to say impossible, to carry on.

Based on the existing studies, it cannot be said that it is an innocuous technique that only cures the disease and does not generate adverse effects. Moreover, there is no available information on the presentation of these side effects which means that we won't be prepared to treat them.

The use of CRISPR / Cas for the genetic edition in embryos will suppose in the future an important therapeutic weapon but today it is still in a very preliminary state. Over time more studies will appear, and we will understand better and therefore control the off-target effects produced by this technology which will allow us to assume the consequences of such effects.

REFERNCES

1. Johnson C, Davidson B. Huntington's disease: progress toward effective disease-modifying treatments and a cure. Human Molecular Genetics. 2010;19(R1): R98-R102.
2. Kleinstiver B, Prew M, Tsai S, Topkar V, Nguyen N, Zheng Z et al. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature. 2015;523(7561):481-485.
3. Kordasiewicz H, Stanek L, Wancewicz E, Mazur C, McAlonis M, Pytel K et al. Sustained Therapeutic Reversal of Huntington's Disease by Transient Repression of Huntingtin Synthesis. Neuron. 2012;74(6):1031-1044.
4. Mojica F, Diez-Villasenor C, Soria E, Juez G. Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. Molecular Microbiology. 2000;36(1):244-246.
5. Zetsche B, Gootenberg J, Abudayyeh O, Slaymaker I, Makarova K, Essletzbichler P et al. Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. Cell. 2015;163(3):759-771.
6. Capecchi M. Altering the genome by homologous recombination. Science. 1989;244(4910):1288-1292.
7. Danna K, Nathans D. Specific cleavage of simian virus 40 DNA by restriction endonuclease of Hemophilus influenzae. Proceedings of the National Academy of Sciences. 1971;68(12):2913-2917.
8. Kelly T, Smith H. A restriction enzyme from Hemophilus influenzae. Journal of Molecular Biology. 1970;51(2):393-409.
9. Lin F, Sperle K, Sternberg N. Recombination in mouse L cells between DNA introduced into cells and homologous chromosomal sequences. Proceedings of the National Academy of Sciences. 1985;82(5):1391-1395.
10. Rouet P, Smih F, Jasin M. Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Molecular and Cellular Biology. 1994;14(12):8096-8106.
11. Scherer S, Davis R. Replacement of chromosome segments with altered DNA sequences constructed in vitro. Proceedings of the National Academy of Sciences. 1979;76(10):4951-4955.
12. Seligman L. Mutations altering the cleavage specificity of a homing endonuclease. Nucleic Acids Research. 2002;30(17):3870-3879.
13. Smith H, Welcox K. A Restriction enzyme from Hemophilus influenzae. Journal of Molecular Biology. 1970;51(2):379-391.
14. Smithies O, Gregg R, Boggs S, Koralewski M, Kucherlapati R. Insertion of DNA sequences into the human chromosomal β-globin locus by homologous recombination. Nature. 1985;317(6034):230-234.
15. Sussman D, Chadsey M, Fauce S, Engel A, Bruett A, Monnat R et al. Isolation and Characterization of New Homing Endonuclease Specificities at Individual Target Site Positions. Journal of Molecular Biology. 2004;342(1):31-41.
16. THOMAS K. High frequency targeting of genes to specific sites in the mammalian genome. Cell. 1986;44(3):419-428.

17. Choulika A, Perrin A, Dujon B, Nicolas J. Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of Saccharomyces cerevisiae. Molecular and Cellular Biology. 1995;15(4):1968-1973.

18. Faruqi A, Seidman M, Segal D, Carroll D, Glazer P. Recombination induced by triple-helix-targeted DNA damage in mammalian cells. Molecular and Cellular Biology. 1996;16(12):6820-6828.

19. 17 Felsenfeld G, Davies D, Rich A. FORMATION OF A THREE-STRANDED POLYNUCLEOTIDE MOLECULE. Journal of the American Chemical Society. 1957;79(8):2023-2024.

20. 18 Mansour S, Thomas K, Capecchi M. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature. 1988;336(6197):348-352.

21. 19 Rong Y. Gene Targeting by Homologous Recombination in Drosophila. Science. 2000;288(5473):2013-2018.

22. 20 Rouet P, Smih F, Jasin M. Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Molecular and Cellular Biology. 1994;14(12):8096-8106.

23. 21 Strobel S, Dervan P. Single-site enzymatic cleavage of yeast genomic DNA mediated by triple helix formation. Nature. 1991;350(6314):172-174.

24. 22 Strobel S, Doucette-Stamm L, Riba L, Housman D, Dervan P. Site-specific cleavage of human chromosome 4 mediated by triple-helix formation. Science. 1991;254(5038):1639-1642.

25. 23 Varshavsky A. Discovering the RNA Double Helix and Hybridization. Cell. 2006;127(7):1295-1297.

26. 24 Wang G, Levy D, Seidman M, Glazer P. Targeted mutagenesis in mammalian cells mediated by intracellular triple helix formation. Molecular and Cellular Biology. 1995;15(3):1759-1768.

27. Boudreau R, McBride J, Martins I, Shen S, Xing Y, Carter B et al. Nonallele-specific Silencing of Mutant and Wild-type Huntingtin Demonstrates Therapeutic Efficacy in Huntington's Disease Mice. Molecular Therapy. 2009;17(6):1053-1063.

28. Boudreau R, McBride J, Martins I, Shen S, Xing Y, Carter B et al. Nonallele-specific Silencing of Mutant and Wild-type Huntingtin Demonstrates Therapeutic Efficacy in Huntington's Disease Mice. Molecular Therapy. 2009;17(6):1053-1063.

29. Diaz-Hernandez M. Full Motor Recovery Despite Striatal Neuron Loss and Formation of Irreversible Amyloid-Like Inclusions in a Conditional Mouse Model of Huntington's Disease. Journal of Neuroscience. 2005;25(42):9773-9781.

30. Garriga-Canut M, Agustin-Pavon C, Herrmann F, Sanchez A, Dierssen M, Fillat C et al. Synthetic zinc finger repressors reduce mutant huntingtin expression in the brain of R6/2 mice. Proceedings of the National Academy of Sciences. 2012;109(45): E3136-E3145.

31. Harper S, Staber P, He X, Eliason S, Martins I, Mao Q et al. RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model. Proceedings of the National Academy of Sciences. 2005;102(16):5820-5825.

32. HICKS R, SMITH D, LOWENSTEIN D, MARIE R, McINTOSH T. Mild Experimental Brain Injury in the Rat Induces Cognitive Deficits Associated with Regional

Neuronal Loss in the Hippocampus. Journal of Neurotrauma. 1993;10(4):405-414.

33. 6. Gasiunas G, Barrangou R, Horvath P, Siksnys V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proceedings of the National Academy of Sciences. 2012;109(39): E2579-E2586.

34. 9. BELLAVER CAPELLA V. LA Revolución de la edición genética mediante CRISPR-Cas 9 y los desafíos éticos y regulatorios que comporta. 2nd ed. Cuadernos de Bioética XXVII. València; 2016.

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