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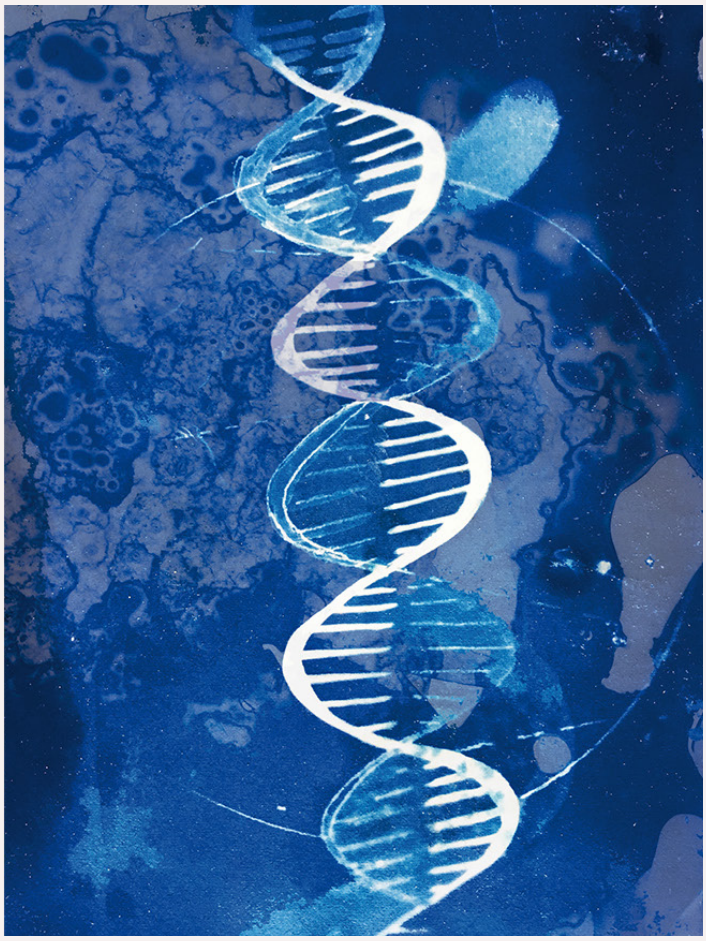
A Report by the Biological Sciences Section of the Institute of Catalan Studies



Institut d'Estudis Catalans

SECCIÓ DE CIÈNCIES BIOLÒGIQUES

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A Report by the Biological Sciences Section of the Institute of Catalan Studies

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PREAMBLE

Since the inception of molecular biology, the new techniques developed by this science and the alterations they allow researchers to make to the human genome have been a cause of concern and ethical debate. This list of techniques is long and includes the restriction enzymes that facilitated the genetic engineering methods developed in the 1970s, the recombinant proteins in animals and plants produced in the 1980s, the viral vector therapies manufactured in the 1990s, and, more recently, new techniques of genomic editing. In 1978, in his book "The Beginning and the End", Isaac Asimov predicted that "The advance of genetic engineering makes it quite conceivable that we will begin to design our own evolutionary progress." Yet, while 40 years ago, the prospect of human gene editing was still little more than a utopian dream, today it has become the most promising and, at the same time, the most controversial technique in modern biology.

Genomic editing opens up hitherto unimaginable possibilities thanks, largely, to Francisco Mojica's 1993 discovery of the clustered regularly interspaced short palindromic repeats (CRISPR) system that prokaryotes have evolved as an effective defense system to fight invasive viruses. Subsequently, other researchers identified the components of the CRISPR system that have made it possible to fine-tune a methodology for editing eukaryotic genes and for ushering in the technological revolution that was to follow. The use of the CRISPR system as a genomic-editing method associated with the Cas9 protein was first described in 2012 and it has since become the most versatile genetic engineering tool created in the recent history of molecular biology. The tool means that any gene can be eliminated, activated, inactivated and corrected, generating a diversity of applications not only in basic research but also in biomedicine, agriculture and livestock ranching. In turn, it opens up the possibility of developing treatments for healing genetic diseases that currently have no known effective therapies. Unsurprisingly, the potential this system has for editing genomes of different species, with unprecedented ease, has

generated much discussion in the scientific community and the system's possible applications and their consequences have led to a major ethical and moral debate in society as a whole.

The CRISPR system can be adapted to edit any DNA sequence and, indeed, this technological revolution is already a reality in many biology laboratories and in those of its two related disciplines: biomedicine and biotechnology. It is also becoming a reality in applications with plants, animals, fungi and bacteria — especially those intended for food — aimed at improving their production, health and adaptation to the environment. And the hope is that the same will soon be true for hospitals allowing them to treat specific mutations in genes that are responsible for some 10,000 congenital monogenic diseases, once, that is, the DNA repair systems required for completing the editing process are fully understood and controlled.

In 2015, the Princess of Asturias Prize for Scientific and Technical Research was awarded to Emmanuelle Charpentier and Jennifer Doudna, for developing a genome-editing technology that enables the genome to be rewritten and defective genes of all cell types to be corrected very economically with an unprecedented level of precision. Unfortunately, however, the jury overlooked Francisco Mojica's contribution. Subsequently, other leading prizes have been awarded to researchers who are leading the way in the development of these new methodologies, culminating with Charpentier and Doudna receiving the 2020 Nobel Prize in Chemistry for the development of a method of genomic editing based on the CRISPR prokaryotic defense system.

Clinical trials employing this technique are currently underway to treat a wide variety of diseases. However, despite significant social support for its therapeutic applications, there is growing concern with regard to the ethics (morality) and safety of using the technique, especially CRISPR applications in germ cell genome editing. Interestingly, this debate was initiated at a meeting in Napa Valley in 2015, when the leading group of scientists working with CRISPR-Cas9 met to examine the legal and ethical concerns of this methodology. Since that date, more extensive deliberations have been held around the world, with the aim of determining when, where and how this technology might be applied in humans.

During the academic year 2019-20, the Biological Sciences Section of the ***Institut d'Estudis Catalans*** thought it apposite to review this ongoing debate in an effort at outlining a number of basic recommendations that might serve for future applications of these techniques. To this end, we organized a series of seminars, led by experts that clearly excel in these methodologies, to address a range of topics in genome editing that include somatic and stem cell editing; genome editing in plants, animals, and humans; applications in human therapy; germinal and embryo genome editing; and the ethical, economic, and social aspects of genome editing.

There are contrasting precedents for the social acceptance of somatic cell DNA modification aimed at providing palpable medical benefits, with a favorable benefit-risk ratio. Examples include solid organ transplants and donor bone marrow cell replacement, that is, different methodological variants of gene therapy for the treatment of serious disorders. In such instances, regulatory policies have been developed, with country-specific variations responding to different cultural and religious beliefs, to ensure that these procedures are carried out in accordance with ethical and social principles. In contrast, the necessary social consensus has yet to be reached for the use of these techniques to modify the germline, a controversial process since it results in a permanent change to the genome that can be passed down through generations. There are countries that ban the process altogether and others that have erected barriers to the use of embryos in different periods of development. For example, 29 countries have signed and ratified the Oviedo Convention (https://www.coe.int/en/web/bioethics/oviedo-convention), which specifically prohibits the editing of the heritable genome. The U.S. National Institutes of Health (NIH) decided in 2015 that they would not fund the use of gene editing technologies in human embryos, arguing that altering the human germline is a line that should not be crossed. Indeed, the first report on the modification of the DNA of two human embryos using the CRISPR-Cas9 technique, published by Chinese scientists in 2015, served as a wake-up call for the scientific community who, shortly afterwards, called a summit meeting of the Chinese Academy of Sciences, the Royal Society, the National Academies of Sciences of the United States and research institutes from other countries. The meeting culminated in a joint statement that included the following statement: "It would be irresponsible to proceed with any clinical use of germline editing unless and until the relevant safety and efficacy issues have been resolved." The statement also highlighted the need for proper regulation and oversight. However, in 2018, He Jiankui, a Chinese scientist, modified the CCR5 gene, encoding a protein that some common strains of HIV use to infect immune cells, in the embryos of twin girls born with edited genomes. His actions, for which he was fined and imprisoned, were condemned as being misguided, premature, unnecessary and largely useless.

If we hope to advance in any scientific field, new methods are needed – as Sydney Brenner famously said: "Progress in science depends on new techniques, new discoveries and new ideas, probably in that order." Likewise, moral and ethical decision-making needs to evolve in parallel with scientific advances, and we recognize that it is entirely reasonable for national and supranational legislations to want to deliberate the regulation of these techniques based firmly, however, on evidence from CRISPR applications aimed at improving the health and promoting the progress of all people.

Ramon Bartrons

Jaume Bertranpetit

EXECUTIVE SUMMARY

Genome-editing techniques make it possible to modify the genome and, hence, the traits of living things with a precision that was unimaginable just a few years ago. More and more innovative applications of these techniques are being described in biomedical research and, increasingly, they are being used to develop new industrial products and medical treatments that would not otherwise be viable. Moreover, the impact of genome-editing techniques is being felt across all domains of the life sciences, from bacteria to humans. Such is the transformative potential of genome engineering applications that it has become imperative that we examine the benefits and risks of their use and debate the societal and ethical implications that derive from their adoption.

At the molecular level, the most widely used genome-editing techniques employ nucleases (proteins with enzymatic function) to recognize a specific genomic sequence and cut it, causing a double-stranded break in the genomic DNA. In response to that break, the cell's DNA repair mechanisms are activated to repair the cut and maintain the integrity of the genome, but in doing so they may introduce mutations or even sequences of interest at the cut site. Several targeted nucleases (including ZFNs and TALENs) have been developed, but it was the repurposing of the CRISPR-Cas9 system as a genome-editing technique in 2012 that initiated the exponential growth in the popularity of these techniques. Unlike these other techniques, the CRISPR-Cas9 system can be used for the low-cost editing of almost any genomic region and, moreover, it allows a large number of genomic regions to be altered at once.

These technical advantages have facilitated the adoption of the CRISPR-Cas9 system as a powerful tool for biomedical research. The possibility of programming CRISPR-Cas9 to alter the expression of thousands of genes has been harnessed to identify the genes responsible for certain phenotypes and diseases. Similarly, the application of genome-editing techniques in an individual's stem cells (whether embryonic or induced pluripotent, or in their organoids) has resulted

in disease models that are being used to develop new drugs and test personalized treatments. Moreover, the genetic modification of embryos has improved understanding of human development and cell plasticity, facilitating advances in the field of regenerative medicine.

The applications of genome-editing techniques are not solely limited to research, but are also being extensively used to improve industrial processes involving microorganisms. Through the activation or repression of multiple genes, genome-editing techniques can significantly alter the metabolism of these microorganisms. In this way, the flow of nutrients can be redirected to obtain a higher yield of a product or even to produce new metabolites of interest. This approach has been used in *Escherichia coli*, other industrially relevant bacteria, fungi, and microalgae to produce complex bioactive compounds, phytochemicals, and biofuels, among many others, including drugs of high interest.

The impact of genome-editing techniques, such as CRISPR-Cas9, on the food sector is also of particular relevance, as it facilitates the production of genetically modified plants and animals. In the case of plants, genome-editing techniques accelerate the process by which new varieties can be obtained, thanks to their ability to direct the mutations to a specific genomic region, avoiding the longer, more tedious process of introducing random mutations and then selecting the varieties with the phenotype of interest. The most common objectives of genetic modifications are to improve plant tolerance to abiotic (e.g. drought) and biotic (e.g. insect pests) stresses, and to enhance their nutrient utilization and nutritional properties. The cultivation of genetically modified plants for food is limited by the regulations and perceptions prevailing in each country. The permissive regulations of the United States, for example, contrast with those in Europe, which strictly control the use of genome-editing techniques. Spain, however, stands out for the high number of transgenic crops that it cultivates compared to other European states, where adoption is virtually null.

In the case of animals, a number of countries to date have approved applications to use a genetically engineered salmon for food. The fish has been modified in order to accelerate its growth without altering its final size, and we can expect more proposals to alter the nutritional properties of various species in the coming years. Beyond these uses in the food sector, genome-editing techniques are also being used in model organisms (*C. elegans, Drosophila*, zebrafish, and mouse) in genetics and developmental biology and to create disease models. However, there are certain applications in animals that, albeit of great potential, raise ethical concerns, such as xenotransplantation, the de-extinction of species, and the gene drive (necessary for the dissemination of new genetic variants); not to mention uses that seek to satisfy purely recreational or aesthetic goals.

A further area of interest is the use of gene-editing techniques to cure diseases and, in fact, the therapeutic application of these techniques in humans is an imminent reality. The first clinical trials are primarily *ex vivo* therapies, in which cells are edited outside the body to produce a therapeutic factor and then transplanted into the patient, or *in vivo* therapies that target accessible tissues such as the eye retina. The use of these techniques is currently being studied to treat blood diseases, such as sickle cell anemia and beta-thalassemia, to boost lymphocytes that eliminate cancer cells, and to treat ocular genetic diseases, among others. Given the current limitations of the vectors that deliver the DNA editing enzymes to the target cells, in the short term it seems probable that somatic therapy will be limited to these cases.

Editing the human germline is of great interest because, unlike somatic editing, which is restricted to a few cells, it would make it possible to edit most, if not all, tissues affected by a disease. Yet, while interesting from a therapeutic point of view, it implies that the individual's germline transmits the mutations to offspring, thus multiplying the effects and risks of the genetic manipulation and compromising the ethics of what is currently considered acceptable. Its present technical limitations prevent the safe use of embryo editing for reproductive purposes, which is why it is banned or restricted worldwide. However, this did not prevent the birth of the first genetically modified babies in China in November 2018. The news illustrated the complexity of strictly regulating the uses of genome-editing techniques and the need for open discussions of the consequences of such experiments before they are carried out. Similarly, there is a pressing need to study the social implications of improving human qualities through genome-editing techniques, since drawing the line between what constitutes therapy and what constitutes improvement is, in certain instances, highly complex.

In short, the applications of genome-editing techniques are both innovative and promising and it will be fascinating to monitor their development in the coming years. Advances to date have exceeded expectations of just a decade ago and applications with major social and ethical implications have already been developed. It seems likely that this trend will continue in the future and it is more than probable that moral issues which we are unable to anticipate today will emerge. It is of paramount importance that, while awaiting the changes that will undoubtedly come in this field, we do not stand idly by but take an active role in disseminating this knowledge to stimulate an informed and plural debate. This is precisely the aim of the series of conferences on genome-editing techniques organized between 2019 and 2020 by the Biological Sciences Section of the ***Institut d'Estudis Catalans*** and which this document aims to summarize and make available to the general public.

1. INTRODUCTION

The genome is the complete set of DNA in an organism, including all its genes and all the elements that regulate them. The human genome is made up of more than 3 billion base pairs (3×10^9) of DNA, and a and two copies of the genome are contained within each nucleated cell of the body (exept for gametes which only have one copy). The DNA sequence of each copy of the genome stores all the information needed to build and maintain an organism. This is why, for instance, a complex multicellular organism can be developed from a single cell (the zygote) with a pair of copies of the genome.

The genome is responsible for organizing cellular behavior, from the simplest metabolic reactions to the most complex signaling pathways. In the case of animals and plants, the genome also orchestrates the regulation of each cell in the context of a tissue and an organ and integrates external stimuli or information. To perform these functions, several proteins interact with genomic DNA specifically in order to regulate gene expression or to use genetic information to synthesize proteins. The DNA sequence largely determines the outcome of these interactions and, in the case of protein coding genes, the sequence of the protein that is eventually obtained. Therefore, the presence of mutations (that is, changes in the DNA sequence) can affect the functionality of the genome and, thus, alter traits of the organism. A good example of this is provided by the set of small genomic variations that determine our physical traits. It is estimated that humans share 99.9% of the genomic sequence and that the remaining 0.1% alone determines our individual characteristics. Note, however, that this 0.1% represents about 3 million nucleotides of the whole genome.

While most mutations have virtually no effect on the functionality of the organism, some may be beneficial and others extremely harmful. For example, a single mutation in the DMD gene can cause dystrophin to malfunction, resulting in Duchenne muscular dystrophy. Likewise, gene mutations are the cause, among others, of such disorders as albinism, sickle cell anemia, cystic fibrosis,

and Huntington's disease. In addition, some conditions, such as hypertension, arteriosclerosis and schizophrenia, are the result of variants in several different genes and their interaction with external factors. These are the so-called complex characters with multifactorial inheritance.

Although these disorders and conditions mean the term mutation typically has a negative connotation, it should not be forgotten that the occurrence of random mutations forms part of the natural dynamics of genomes. Changes in genomes over generations are fundamental to life as we know it, since the rich diversity of species and their unique characteristics have their origin in the new variants produced by processes of mutation. It is estimated that each of us carries about 60 new mutations, 30 from each parent. In addition, a significant part of the genome corresponds to fragments of genomes from other organisms that have been integrated into our human genome and passed down through the generations. Therefore, we are all mutants and transgenic organisms, and we all carry a wealth of new genomic information that could determine some of our biological characteristics.

Genome-editing techniques are the tools that, for the first time, allow us to alter the genomic information of organisms in a very precise fashion. The deliberate introduction of mutations into specific genomic regions in turn facilitates the study of the function of these genomic regions. The combination of this knowledge and the genome-editing techniques opens the door to the rapid, targeted modification of an organism's characteristics. This alone explains the exponential increase in applications of genome-editing techniques across the domains of the life sciences.

2. GENOME-EDITING TECHNOLOGIES

Recent advances in gene- and genome-editing methods have had a major impact on both basic and applied research and while the importance of a living being's DNA has been known now for over 60 years, these methods have made it possible to modify DNA and improve our understanding of the information it encodes. This section undertakes a review of the development of genome-editing techniques, starting with the first DNA modification methods and ending with the CRISPR-Cas9 system and its variants.

2.1. Genome editing before CRISPR-Cas9

The earliest techniques developed to insert new genetic elements at specific points in the genome were based on the homologous recombination mechanism that occurs naturally in some cells. To achieve this, the DNA sequence of interest is flanked by the genomic sequence of the site in which it is to be integrated and inserted into the cell. Then, it is the cell's own DNA repair mechanisms that use the ends of the introduced DNA sequence to perform recombination and integrate the sequence of interest into the genome. Although this technique has been used to create multiple strains of mice, its efficiency is relatively low[1].

The efficiency of inserting exogenous sequences into the genome can be improved if a double- stranded break (DSB) is made at the point of insertion. In response to this cut, the damaged DNA is repaired to maintain its integrity by homology directed repair (HDR) or non-homologous end joining (NHEJ) mechanisms[2]. In the case of HDR, the cut is repaired from a sequence of nucleotides homologous to the broken ends. If the sequence used as a model is that of the sister chromatid, the cut is repaired by restoring the original sequence. However, if an exogenous DNA sequence with homologous ends has been introduced (as in the technique described above), HDR mechanisms can use it as a template. Thus, the genome would incorporate the exogenous sequence right into the region specified. This

method allows genomic regions to be changed for a sequence of interest with great precision. In contrast, in NHEJ, the cut is repaired by tying the ends adjacent to the cut point without the use of a homologous template. Therefore, the repair result contains small insertions and deletions (indels) of various sizes. The two repair pathways are useful for editing the genome, but the results obtained differ (Figure 1). While HDR inserts a specific modification, NHEJ generates multiple, diverse indels. Both strategies can compromise the functionality of a genetic element, so both pathways of repair are of interest for genome editing.

The possibility of making DSBs in DNA became feasible from the 1990s onwards with the identification and characterization of meganucleases, which can recognize a very long DNA sequence (up to 40 nucleotides) as the cut site[3]. The recognition site is long enough to provide sufficient specificity to cut a genome at a single target point, even in complex genomes such as those of humans. However, the main limitation in using meganucleases as genome-editing techniques is their identification of the specific target region of interest as the cut site. Despite the presence of hundreds of meganucleases in nature and the development of methods

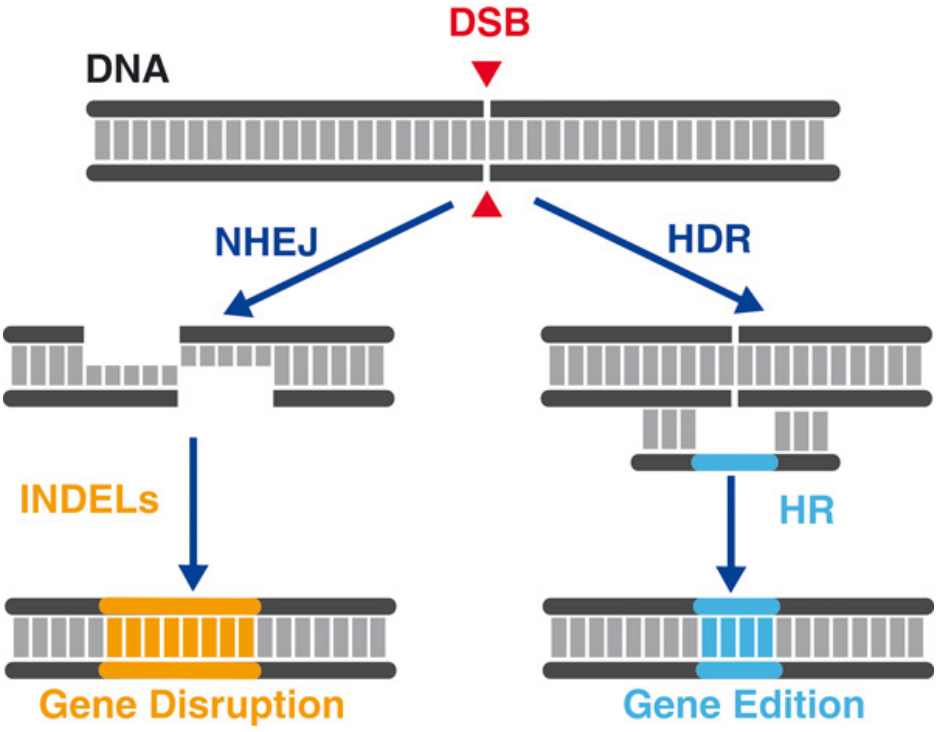

Figure 1. *Double stranded-break repair pathways. Non-homologous end joining (NHEJ) generates insertions and deletions (indels) that disrupt the function of a genomic element. In contrast, in homologous directed repair (HDR), part of the gene is changed for a sequence of interest. Adapted from Lluís Montoliu, 2019*[4].

that allow their structure to be modified to change the cut site, their use in genome editing is not very common because of the simplicity of alternative methods.

Targeted nucleases solve the limitations of meganucleases because they can be designed to bind to any DNA sequence, allowing the introduction of a DSB into any genomic region of interest. Targeted nucleases include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) (Figure 2), and the CRISPR-Cas system.

ZFNs are composed of a DNA binding domain, consisting of three to six zinc fingers, and a DNA cleavage domain, formed by the non-specific cleavage domain of FokI nuclease[5]. Each zinc finger recognizes three nucleotides of DNA, so they can be combined to design a unique ZFN binding point in the genome. Thus, zinc fingers determine the binding of ZFN to a specific DNA sequence, directing FokI nuclease to introduce a DSB at a specific point in the genome.

TALENs are similar to ZFNs in that they also combine proteins that bind to DNA to direct FokI nuclease to the cleavage point. In this case, however, DNA-binding proteins are transcription activator-like effectors (TALEs) as opposed to zinc fingers. TALEs recognize a single DNA nucleotide, while zinc fingers recognize three. Therefore, the design of TALENs to introduce double cut-off points in regions of the genome of interest is simpler than that of ZFNs[6].

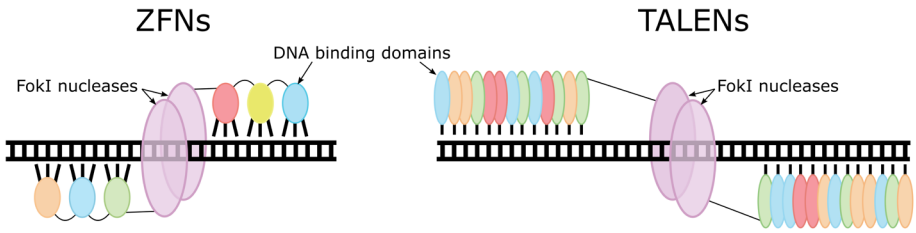

Figure 2. *ZFN and TALEN targeted nucleases. Both ZFNs and TALENs combine several DNA-binding domains to direct FokI nucleases to a target region of the genome and introduce a double-stranded break. While in ZFNs the binding domains are zinc fingers that recognize three nucleotides, in TALENs the binding domains are TALEs that recognize a single nucleotide. Adapted from Beumer and Carroll, 2014*[7].

ZFNs and TALENs have been used extensively in research to generate disease models, both in human stem cells and in model organisms[8], and in plant genome modification to develop variants with improved nutritional properties[9]. They have also been used in various clinical studies. For example, they have been used to delete the CCR5 receptor in CD4 T cells that may confer resistance to HIV, to administer a correct copy of factor IX to treat patients with hemophilia B, and to create T lymphocytes with chimeric antigen receptors (CARs) to treat acute lymphocytic leukemia, among others.

However, since the CRISPR system for genome editing was first described in 2012[10–12], the CRISPR system has gained popularity at a much faster rate than the other targeted nucleases. While ZFNs and TALENs rely on the combination of several proteins to recognize DNA, the CRISPR system uses a ribonucleic acid (RNA) molecule. As it is much easier to design an RNA than the proteins that guide ZFNs or TALENs to a specific region, the CRISPR system is cheaper (just a hundred euros) and faster (a matter of days) to apply than other methods (which can cost thousands of euros and take months). It is precisely the simplicity of the CRISPR system, together with its specificity, efficiency and the possibility it affords of performing many simultaneous editions (multiplexing), that has popularized the use of genome editing in basic research and in applications in fields such as biotechnology, agriculture, and gene therapy.

2.2. The CRISPR-Cas9 system

The CRISPR-Cas system was originally discovered as a prokaryotic adaptive immunity system that confers resistance to foreign genetic elements such as plasmids and viruses. This discovery was critical to the re-programming of the CRISPR-Cas technique as a gene-editing tool later in 2012. Francisco J.M. Mojica proposed the name "CRISPR" to describe a series of short, grouped, and regularly spaced palindromic repeats (clustered regularly interspaced palindromic repeats) that had been described in 1987 in bacteria, and which Mojica identified in archaea in 1993[13] suggesting that the presence of such a similar structure in so many different microorganisms might indicate that CRISPR had an important function for prokaryotes. By 2000, Mojica had identified CRISPR in 20 species of microbes, and two years later the presence of CRISPR-associated (Cas) genes was described. These were found in close proximity to the CRISPR array, and so were considered as having a related function. Interestingly, the name CRISPR appears for the first time in this study undertaken by Dutch scientists, who previously consulted with Mojica as regards the most appropriate name for this unique family of DNA sequences[14] The function of CRISPR was gradually clarified when Mojica observed that the DNA sequences forming the spacers between the CRISPR repeats were identical to the fragments of some plasmids and viral genomes. This led him to suggest that CRISPR loci could encode the instructions of an adaptive immune system that protected microbes from specific infections[15]

Over the following years, the work of multiple authors[16–18] confirmed the function of CRISPR as an adaptive immune system as proposed by Mojica. Briefly, during infection of a genetic element such as a virus or conjugation plasmid, new sequences identical to a fragment of the invasive genetic element are inserted as spacers between the CRISPR repeats of the compromised cell genome (acquisition). They are then transcribed and processed as small

fragments of CRISPR RNA (crRNA) derived from the CRISPR set and which include the spacer sequence identical to an invasive genetic element (expression). These crRNAs then form a complex with the Cas proteins. Thus, the crRNA-Cas ribonucleoprotein complex targets a complementary sequence in the invasive genetic element and cuts it to inactivate it (interference). The CRISPR sequences contain spacers that act as a memory of past infections and, since they are found in the genome, they are transmitted between generations. While CRISPR sequences only store information, Cas proteins are the effectors of the system and are responsible for both storing new sequences of invaders and using the CRISPR memory to recognize and introduce a cut in invasive nucleic acids so that its functionality is inhibited (Figure 3).

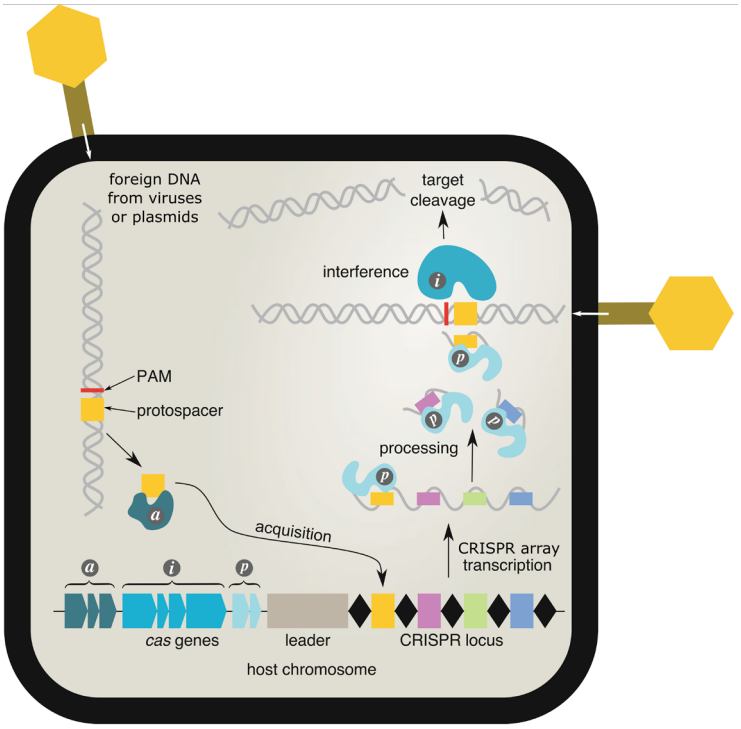

Figure 3. *CRISPR as an adaptive immune system. The CRISPR-Cas system is composed of several cas genes (blue arrows) that express effector proteins involved in the acquisition (a), expression (p), and interference (i) phases of the system. It is also composed of repetitive CRISPR sequences (black diamonds) separated by spacers (multiple colors), the sequence of which is identical to fragments of invasive elements and has been incorporated into the acquisition process. The CRISPR locus is preceded by a promoter (leader) that allows the spacers to be transcribed and processed to generate the crRNA. In the inference process, crRNA-Cas complexes recognize and cut sequences of invasive genetic elements complementary to crRNA. Adapted from Mojica and Garrett, 2013*[14].

At this stage, the potential of the CRISPR system was more than apparent, but there was still some way to go before it would achieve the level of interest it currently enjoys as a genome-editing technique. Indeed, a number of developments were required to adapt CRISPR. First, it was important to demonstrate that the *Streptococcus thermophilus* CRISPR system only used the Cas9 protein to recognize and cleave nucleic acids complementary to crRNA[16]. In addition, the induced cut is blunt, double-stranded, and always in the same position, that is, three nucleotides from the sequence of the motif adjacent to the proto-spacer (PAM). The PAM sequence, which had already been described, is a motif found in the genome near the point of cleavage of Cas proteins and is essential in enabling them to bind to DNA and cleave it. Different Cas proteins recognize different PAMs. For example, the *Streptococcus pyogenes* Cas9 protein (the most widely used today) recognizes the "NGG" PAM, where "N" can be any nucleotide.

Next, Emmanuelle Charpentier showed that Cas9 needs both crRNA (which contains the sequence complementary to the genetic element to be cut) and a CRISPR trans-activating RNA (tracrRNA) to have nuclease activity. Then, having characterized all the elements required for the nuclease activity of Cas9, the team formed by Charpentier and Jennifer Doudna demonstrated that the system could be used to cut DNA sequences *in vitro*[10]. In addition, they simplified the system to just two elements: the Cas9 protein and a single-guide RNA (sgRNA, commonly abbreviated to guide RNA (gRNA)), which is a fusion of crRNA and tracrRNA (Figure 4). A few months later, the teams of Feng Zhang and George Church adapted the CRISPR-Cas9 system to perform genome editing in mammalian and human cells[11,12].

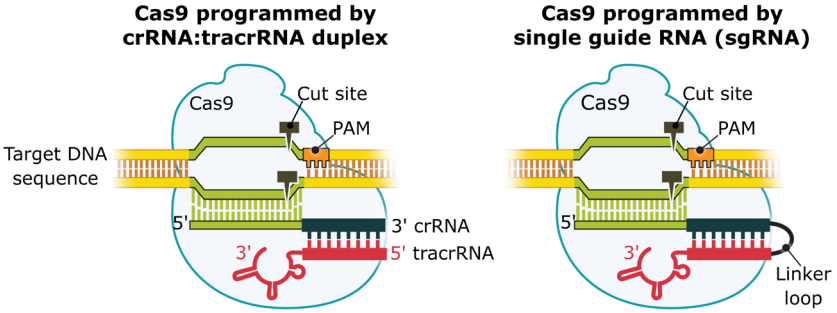

Figure 4. *Recognition of the DNA region to be cleaved by the S. pyogenes Cas9 complex. The Cas9 protein binds to a DNA sequence that is complementary to an RNA molecule. The RNA molecule determining the binding point may be either the crRNA:tracrRNA duplex (left) or a fusion of the two known as sgRNA (right). The PAM sequence is found on the DNA sequence that is to be cut, three nucleotides downstream of the cut point. Adapted from Doudna and Charpentier, 2014*[19].

The work of Charpentier, Doudna, Zhang, and Church was instrumental in making the CRISPR-Cas9 system the first programmable genome-editing technique to be directed by an RNA molecule. Its mechanism of action, moreover, is quite straightforward: it simply requires the design of a gRNA that is complementary to the target region in which the cut is to be introduced, its synthesis, and its introduction together with the Cas9 protein into the cell to be edited. This simplicity, together with its versatility and low cost, has facilitated the rapid adaptation of genome editing with CRISPR-Cas9 to other organisms and popularized its use in research and technological development.

2.3. Rapid advances derived from the CRISPR-Cas9 system

Since the first application of the CRISPR-Cas9 system as a genome-editing technique, basic research has advanced rapidly and created new genome-editing systems derived from CRISPR. For example, protein engineering has been used to modify the Cas9 protein to improve editing efficiency and to perform functions that do not require having to cut DNA, such as regulating gene expression. The properties of CRISPR systems of prokaryotes that differ from the *S. pyogenes* CRISPR-Cas9 system have also been explored and this means genome editing can now directly edit RNA, for example.

Many advances have focused on improving the specificity and efficiency of CRISPR-Cas9 DNA cleavage. Although the 20 nucleotides of sgRNA allow Cas9 to be directed to a specific point in the genome, CRISPR may cut untargeted regions of the genome (off-target editions), especially if these have a sequence that is similar to the target site. The frequency of off-target editing has been reduced thanks to computational tools that help the researcher design highly specific gRNAs to edit a certain gene. Optimization tools have also been used to obtain more specific Cas9 variants[20,21]. A further challenge faced when using *S. pyogenes* Cas9 is the need to have a PAM with an "NGG" sequence adjacent to the cut site since this limits the genomic positions where a double-stranded break can be introduced. Therefore, variations of Cas9 have been created that relax the requirements for the PAM sequence, such as recognizing the "NG" as opposed to the "NGG" motif in order to increase the genomic regions that can be edited.

One of the most widely used variants of Cas9 is dead Cas9 (dCas9), which does not cause a double-stranded break but maintains the specificity that directs dCas9 to a specific region of the genome. Base editors take advantage of this property by fusing dCas9 to an enzyme that chemically modifies nucleotides. Thus, cytosine base editors (CBEs) can modify cytosine to thymine at a specific point in the genome[22], while adenine editors (ABEs) convert adenine to guanine without cutting the genome at any point[23]. Similarly, prime editing fuses a Cas9

that cuts a single strand of DNA to a reverse transcriptase in order to copy a template sequence specified in the gRNA into the genome. This allows the precise modification of the editing site by inserting specific insertions, deletions, or modifications. The development of base editors and prime editing allows researchers not only to control the region to be modified, but also to control with great precision the mutation that is introduced[24]. This means these techniques have great potential for use as gene therapies, since they should one day be able to repair up to 90% of the genetic variants associated with human diseases.

The dCas9 variant has also been used to regulate gene expression. The binding of dCas9 alone to a regulatory DNA region prevents other proteins (such as transcriptional activators) or RNA polymerase from binding to it. Thus, dCas9 can be targeted to regions of the genome to determine whether they are involved in regulating gene expression, or to regulatory regions already known to under or overexpress a genetic element. These approaches are known as CRISPR interference (CRISPRi) or CRISPR activation (CRISPRa), depending on whether gene activity is activated or repressed, respectively. dCas9 can even be fused with transcriptional activators such as VP64 or repressors such as KRAB to enhance its effect[25]. The ease with which CRISPR can be used in multiplex tests allows the effects of the change in expression of many genes to be determined simultaneously and this has facilitated considerable advances in the characterization of the genetic function and in our understanding of the mechanisms of gene regulation.

In addition to creating variants of *S. pyogenes* Cas9, CRISPR systems of other prokaryotic organisms with completely different properties have been studied and used. CRISPR-Cas systems can be classified into two classes, depending on whether the DNA binding and cleavage activity is performed by a complex of several subunits (Class 1) or a single protein (Class 2). For example, *S. pyogenes* has a Class 1 CRISPR-Cas system because the Cas9 protein alone binds and cleaves DNA, while *E. coli* has a Class 2 system because the binding activity and cut is made by a protein complex known as Cascade. Most studies use Class 2 enzymes, as the expression of multiple subunits required by Class 1 can be more complex. However, some Class 1 CRISPR-Cas systems have been used to edit human cells and plants as they can have certain advantages in some applications[26]. For example, the study of CRISPR-Cas systems encoded in transposons has demonstrated that several CRISPR-Class 1 case subunits can fuse with transposon integrases to direct with a guide RNA the insertion of long DNA fragments into specific regions of the genome, which could be an important tool for gene therapy[27].

The diversity of CRISPR-Cas systems is even greater among Class 2 systems, which encompass type II, V and VI systems[28]. For example, Cas9 of *S. pyogenes* is type II and causes cuts at the same point in each of the DNA strands (blunt cuts),

while Cas12a of type V (formerly known as Cpf1) cuts DNA at different points in each chain. Even more surprising is the type VI Cas13 protein, which instead of binding to DNA molecules binds to RNA molecules and has been used both to prevent gene translation and to introduce modifications directly in RNA.

In summary, the design of fusion proteins with dCas9 and the characterization of alternative CRISPR-Cas systems are providing new tools for genome modification with a range of interesting properties for research, industry, and gene therapy. These, added to the simplicity and versatility of the CRISPR-Cas system9, mean it is foreseeable that CRISPR-Cas-derived systems will be used in the coming years in many diverse areas to address problems that until now have proved impossible to solve.

3. GENOME EDITING IN BIOMEDICAL RESEARCH

Genome-editing technologies are both a tool for discovery and a potential solution to the fundamental problems of human genetic diseases. The manipulation of genes and their expression has been applied to human cell lines, tissues, gametes, and embryos to better understand their biochemical mechanisms, including why they function incorrectly in certain diseases. For instance, models of the genetic diseases or genetic changes that occur during cancer can be recreated to identify the molecular basis of these alterations and to test drugs to treat them. Genome editing can also be used to investigate the process of stem cell differentiation, improving our knowledge of regenerative medicine and human embryonic development. The use of genome-editing techniques in induced pluripotent stem cells (iPSCs) and organoids has been responsible for an unprecedented advance in the field of biomedical research of human diseases.

3.1. CRISPR screens

CRISPR screens use CRISPR-Cas9 to simultaneously modify multiple targets in the genome. This is possible thanks to the versatility of gRNA in directing genome editing to any specific region. To achieve this, a library of gRNAs is first created in which each gRNA directs CRISPR-Cas9 to a different region of interest and is introduced (together with the Cas9 enzyme) into the cells the researcher wants to study. The result is a population of cells edited in different genomic regions, which can then be used to determine the effect of genome edits using a phenotypic test. In this way, thousands of genes can be studied to test several genetic hypotheses in parallel (Figure 5).

The use of CRISPR screens has accelerated the identification of genetic elements involved in disease. A good example of this is the study reported by Shalem *et al.* in 2014[29]. In it, a library of more than 60,000 gRNAs is used to direct CRISPR-Cas9 to edit and cause the loss of function (knock-out) of about 18,000 human genes. Because each cell incorporates a different gRNA, a cell population is obtained that allows researchers to test the effect of the loss of function of each of the studied genes. The study used the library to identify genes whose loss of function provides resistance to vemurafenib (PLX), a drug used to treat melanoma. To do this, the library is introduced into a melanoma cell line that is cultured with PLX to inhibit its growth. After a few days of culture, only those cells with a gRNA that causes the loss of function of a gene giving resistance to PLX grow. At the end of the experiment, the gRNAs are sequenced to compare the abundance of each in the cells at the beginning and the end of culture. The enriched gRNAs are those of the cells that have grown and, therefore, the regions targeted by these gRNAs are the ones involved in PLX resistance. Thus, the mechanisms of PLX resistance can be clarified and melanoma treatments improved.

Although the initial application of CRISPR screens was in loss-of-function assays, the technique has been adapted to study the effects of change in gene expression (both overexpression and repression) and the modification of genetic enhancers. It can also be adapted for epigenomic studies by altering DNA methylation patterns and chromatin status by histone compaction[25]. This versatility and ease of testing thousands of conditions has led to the use of CRISPR screens in the study of both cancer and inherited genetic diseases and infectious agents.

When it comes to the treatment of cancer, precision genomic medicine is one of the most promising strategies given the importance of genomic variations caused by the genomic instability of cancer cells. CRISPR screens have been used to identify the genomic variations responsible for proliferation, whether they are variants that mutate a protein or alter gene expression. The method increases understanding of cancer at the molecular level and facilitates the identification of genetic elements that could be targeted by drugs to treat cancer. CRISPR screens have even been used to study the resistance to drugs that some tumor cells develop, as shown in the example above, with the goal of combining more than one drug to avoid resistance.

In the context of congenital genetic abnormalities, CRISPR screens have been used, for example, to identify regulators of the expression of different forms of hemoglobin. Hemoglobin disorders, such as beta-thalassemia or sickle cell anemia, are relatively common and are caused by defects in the adult form of hemoglobin (hemoglobin A). Before birth, the main oxygen transporter is not hemoglobin A but fetal hemoglobin. Natural variations that cause the expression

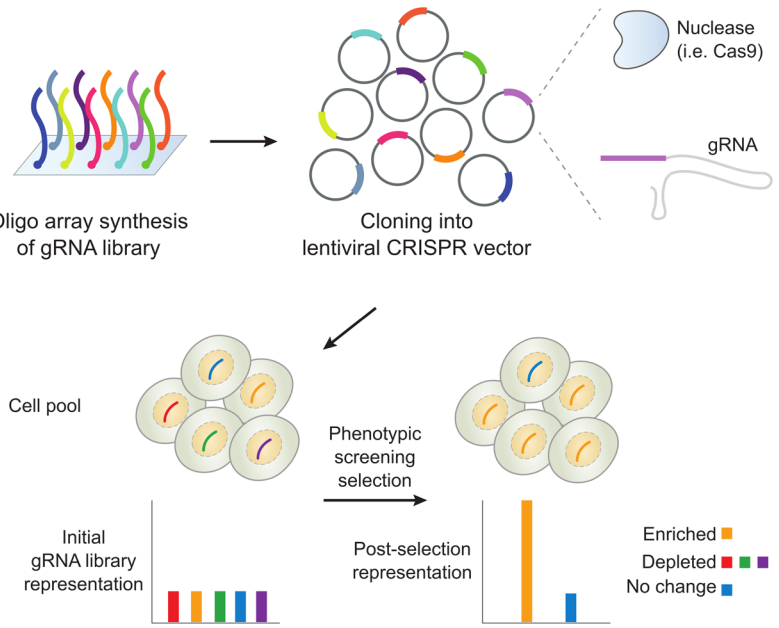

FIGURE 5. *Schematic representation of a CRISPR screen. In a CRISPR screen, cells are transfected with a library of gRNAs that target many genomic regions and a CRISPR nuclease to edit these regions. Then, a phenotypic selection test is used to select the set of cells that presents the phenotype of interest, which will have the gRNAs that target the genomic regions responsible for it. Finally, the change in the abundance of each gRNA is assessed to identify the genetic elements associated with the phenotype of interest. Adapted from Sanjana, 2017*[30].

of fetal hemoglobin components in adults have been shown to prevent the serious consequences of the most common hemoglobin A disorders. Therefore, CRISPR screens have been used to identify regulatory elements that control the expression of various forms of hemoglobin[31]. Thus, drugs could be designed to inhibit or activate regulatory genes or genome editing could be directly used to activate the expression of alternative forms of hemoglobin in order to alleviate the effects of the disorders.

In the case of infectious agents such as bacteria, parasites, and viruses, CRISPR screens have been used primarily in the host to identify factors that make it vulnerable or resistant to infection. A good example of this are various studies that have identified genes involved in the degradation of proteins essential for the infection and replication of flaviviruses such as dengue[32]. The characterization of factors that make a host resistant or sensitive to infection offers new options for the development of antimicrobial and antiviral therapies. In addition, in some cases, CRISPR screens have been used directly on pathogens to characterize the mechanisms of action of new antibiotics[33].

In summary, the use of CRISPR screens in biomedical research has improved the understanding of the genetic basis of diseases and led to the identification of new molecular targets for their treatment. The ability to study thousands of editions in a single analytical test provides information that facilitates the use of personalized medicine in the treatment of cancer. In addition, CRISPR-Cas9 variants allow the study of the effects of gene expression and its regulatory mechanisms, providing insights into the large number of disease-associated variants that lie outside genomic regions encoding a gene.

3.2. Genome editing in human stem cells

Human pluripotent stem cells (hPSCs) are widely used in biomedical research because they can be cultured *in vitro* by dividing indefinitely and they can differentiate into any cell type in the human body. Depending on their origin, they can be classified as either embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). The former are obtained from human blastocysts, while the latter are re-programmed from adult somatic cells through transcription factors and small chemical molecules, thus avoiding the use of human embryos. hPSCs can be cultured *in vitro* and differentiated to form neuronal, muscle, or skin cells, among others. The knowledge acquired with these cells and their therapeutic potential form the basis of the field of regenerative medicine, which aims to replace damaged cells in human tissues and generate new tissues to help in the recovery from disease or injury. In addition, hPSCs have certain advantages over animal models that make them especially useful in research.

Genome-editing methods are used to generate a wide range of genetic modifications in human ESCs and iPSCs. The recombination efficiency of human ESCs is lower than that of mice, so traditional homologous recombination-based techniques used in mice are not effective for human ESCs. Therefore, the development of targeted nucleases such as ZFNs, TALENs, and CRISPR-Cas9 has been essential to edit the genomes of hPSCs. In this context, these techniques have been used to create disease models, discover new drugs, individualize treatments, and study human development.

3.2.1. *Disease models*

Despite advances in the development of animal models (such as mice and zebrafish) to recreate human diseases, they cannot fully capture the pathology of some human diseases. In these cases, the use of cell lines extracted from patients as disease models is very useful. iPSCs are especially useful because they can be differentiated into cell types that are difficult to obtain from patients directly,

such as neurons. Thus, they have been used to model human diseases and to understand the mechanism responsible for their pathogenesis[34].

Before modern genome-editing techniques became available, disease models were created by comparing the cells derived from iPSCs of diseased patients and healthy donors. Because the cells came from two different people, the phenotypic and genotypic differences observed could not be fully attributed to the disease, as there are confounding factors such as genetic variability between individuals, age, and sex. Genome-editing techniques solve this problem by allowing the creation of cells derived from patients that differ only in the mutations that cause the disease, known as isogenic (same genetic origin) controls. This technique is applied by introducing the disease-causing mutation in healthy donor cells and by correcting the mutation in patient cells[35]. For example, correction of the G2019S mutation in the LRRK2 gene in neural stem cells differentiated from iPSCs from a Parkinson's patient prevents the onset of nuclear aberrations that characterize Parkinson's disease. Likewise, the introduction of the same mutation in hESCs of a healthy donor induces the same phenotype as that of the cells of the patient with Parkinson's, confirming that the G2019S mutation is necessary and sufficient to cause the phenotype of Parkinson's disease[36]. Isogenic controls such as these have been applied to create models of a wide range of diseases, including Duchenne muscular dystrophy, Tangier disease, and Rett syndrome, and to create allelic variants of CCR5 resistant to the human immunodeficiency virus (HIV)[37]. In addition, the possibility of modifying more than one allele with CRISPR-Cas9 has facilitated the creation of models of polygenic diseases[38].

One disadvantage of disease models based in hPSCs over animal models is that the former cannot reproduce the organism's physiological conditions. To address this, co-culture systems of various cell types in 3 dimensions, such as organoids and organs-on-a-chip, have been developed[39]. Organoids are functional tissue units with diverse cell types and with an organization and function relatively similar to that of an organ in the human body, so they are interesting models of human tissues. As with hPSCs, genome-editing techniques have been used in organoids to create models or correct mutations in diseases such as cystic fibrosis, polycystic kidney disease, and various types of cancer[40,41]. An alternative to using organoids, and one that is gradually gaining proponents, is the incorporation of hPSC-derived cells in animals to build humanized animal models, such as transplanting hPSC-derived hepatocytes into a damaged mouse liver and observing the effects of human genetic variation on the context of the physiological conditions of the mouse[42].

3.2.2. *Drug discovery*

In addition to studying the mechanisms responsible for pathogenesis, hPSC-based disease models can be used to discover new drugs with therapeutic effect. Drug discovery is conducted using one of two possible strategies: testing a candidate drug or using high-throughput screening (HTS). In the case of the first strategy, a small group of defined molecules is tested by comparing their action in a disease model and in a healthy isogenic control. In contrast, in the second strategy, a high number of compounds with small variations are tested and phenotypic effects are assessed automatically using robots. In either case, the candidates selected in the first *in vitro* assay will go on to secondary validation assays to characterize their safety and pharmacokinetic properties (absorption, distribution, metabolism, and elimination of drugs within the body). The use of hPSC-based human disease models improves the efficiency and accuracy of the drug discovery process, as they offer a very direct *in vitro* representation of pathogenic conditions, reduce the number of candidates to be tested in the following phases, and speed up the screening process. In addition, when culture techniques and the high-throughput screening process are automated, models can be developed from iPSCs derived from a particular patient and used to test the effect of drugs on the genotype of the donor patient, which allows the potential of personalized medicine to be employed in clinical practice[43].

hPSC-based models can also be used to assess the toxicity of a drug and to discard it if it is unsafe. In this case, hPSCs do not act as models of disease but as models of a tissue to test if the drug has adverse effects on it. This allows researchers to predict severe adverse effects related to toxicity in certain tissues and to prevent the withdrawal of drugs that are already in use, as has happened on occasions[44]. For instance, it has been demonstrated that cardiomyocytes derived from ESCs can be used to test the cytotoxicity of drugs, with similar results being obtained in the *in vitro* model and pre-clinical animal toxicity studies[45]. Therefore, these models are valid for testing cytotoxicity at an early stage in drug development and facilitate the ruling out of toxic drugs before moving on to animal testing.

3.2.3. *Study of human development*

Despite their biological and clinical importance, the molecular mechanisms governing early cell differentiation decisions are still not fully understood. Genome-editing techniques can be used to modify genes in the zygote stage and to observe the effect of these mutations directly on genetically modified embryos. For example, a 2017 study described microinjection in human zygotes of the Cas9 protein complexed with a gRNA that directs the breakdown of the gene

encoding OCT4, a transcription factor involved in maintaining pluripotency. The results indicate that the loss of OCT4 factor in human embryos prevents the proper development of the blastocyst. The study also identifies differences in the function of OCT4 factor in the early stages of development in human and mouse embryos, emphasizing the importance of testing in human embryos, which differ from mouse embryos in various aspects[46].

It should be noted that human development studies performed on embryos do not involve their implantation or birth, so none of the changes is transmitted to future generations. In addition, the zygotes used are left over embryos from *in vitro* fertilization procedures of couples that opt not to implant them and who have given their informed consent for their use in research. The development of the embryos in the study described above was stopped at the maximum legal limit of 7 days, although in some countries, such as Spain, development is allowed up to 14 days after fertilization. Even if embryo studies are stopped before implantation, experimentation with genetically modified human embryos can provide the knowledge needed to understand the first steps in development and the differences in these steps between humans and other animals[47]. The knowledge gained in these studies should be useful to improve *in vitro* fertilization processes, develop new contraceptive methods, and better understand cell plasticity to advance the field of regenerative medicine. In addition, improved techniques used to genetically modify human embryos could one day be used to treat genetic diseases before birth. Basic research in this area provides essential information on the feasibility of making heritable changes in the genome, such as the importance of off-target editing errors and mosaicism, which are discussed later in this document.

4. GENOME EDITING IN MICROBES

Prokaryotic microorganisms were using the CRISPR-Cas system long before its use became popular among scientists. The CRISPR-Cas system naturally acts as an adaptive immunity system, protecting bacteria and archaea from foreign genetic elements, including plasmids and viruses. However, since its reformulation as a genome-editing technique in 2012, researchers have used it to make precise changes to genomes. As a genome-editing technique, its use is not limited to prokaryotes, having already been used in microbial eukaryotes, including fungi and microalgae, and complex organisms such as plants and animals. This section discusses the applications of genome-editing techniques in bacteria, archaea, fungi, and microalgae.

In this set of microbes, the CRISPR-Cas9 system is the most widely used editing technique thanks to its ability to modify more than one genomic region at a time. This allows the modification of several components of the metabolism of a microorganism – a practice known as metabolic engineering – so that it produces new metabolites of industrial interest or obtains a higher product yield per quantity of substrate (Figure 6). In addition, CRISPR-Cas9-derived techniques, such as base editing or prime editing, facilitate the introduction of precise changes in the genome to improve the properties of the microorganism's own enzymes.

4.1. Applications of genome editing in prokaryotes

Bacteria can use simple, inexpensive raw materials, such as renewable biomass or waste from other processes, to synthesize value-added chemicals. In this way, they can be used industrially as cell factories and their properties are often improved through genome editing. Specifically, the CRISPR-Cas system facilitates the editing of dozens of genomic regions and this has been used to create hundreds of bacterial strains with unique metabolic properties and, also,

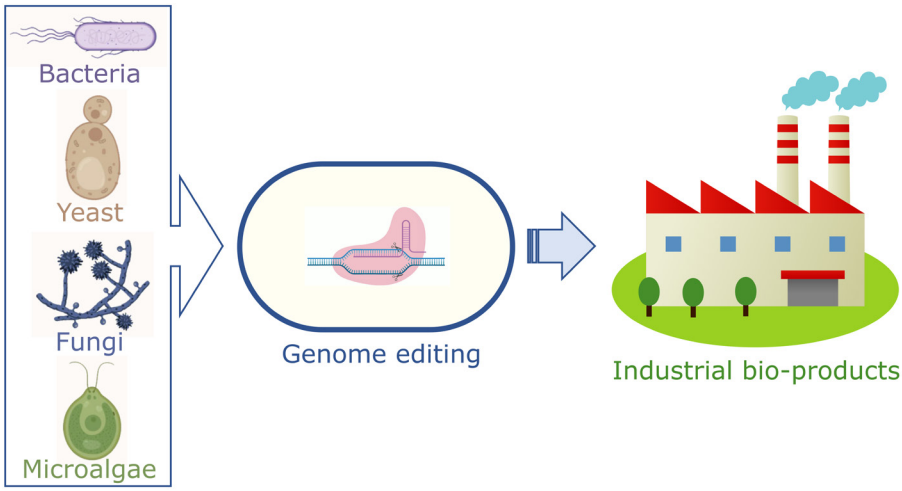

FIGURE 6. *Genome editing of microbes for the production of biological compounds of industrial interest. The metabolism of bacteria, yeasts, fungi, and microalgae can be adjusted using genome-editing techniques (here CRISPR-Cas9 is represented) to improve their industrial application. Adapted from Abdelaal and Yazdani, 2020*[48].

to test them to identify those that improve product yield. The CRISPRi variant, which is used to repress the expression of specific genes, allows the activity of metabolic pathways that are of no interest to be reduced and to redirect the flow of nutrients to the metabolic pathway of interest to boost product yield. These metabolic engineering strategies have been applied in *E. coli* to improve the production yield of biofuels (such as isopropanol), phytochemicals (such as carotene), and polyhydroxyalkanoates (PHAs) that can be used as renewable and biodegradable plastics[49].

The CRISPR-Cas system also facilitates genome editing in organisms of industrial interest that are not as well-known as *E. coli*. One example is the editing of *Clostridium*, a genus that is difficult to modify genetically and which requires efficient editing techniques, such as those afforded by CRISPR-Cas9. Genetic modifications in clostridia have improved the production of biofuels such as butanol from renewable carbon sources. *Streptomyces* are used to produce secondary metabolites with biological activity, including antibiotics and herbicides, while the use of CRISPR-Cas9 has enabled the activation of metabolic pathways that were silenced, thus generating new secondary metabolites that could have interesting biological functions. *Bacillus subtilis* is used industrially to produce recombinant proteins and is also designated by the FDA as "generally recognized as safe" (GRAS). CRISPR-Cas has been used to produce biological compounds of medical interest in *B. subtilis*, such as hyaluronic acid and N-acetylglucosamine. The CRISPR-Cas system has also improved the properties of other bacteria of industrial interest,

including cyanobacteria, lactic acid bacteria (LAB), and species of the genera *Corynebacterium* and *Pseudomonas*[50].

Genome editing in bacteria could also be used to treat bacterial infections. The specificity of genome-editing techniques can be harnessed to edit and kill a particular bacterial strain from a mixed bacterial consortium. This precision offers the advantage of affecting only the pathogenic population while leaving the rest undamaged. For example, CRISPR-Cas9 has been used to cut a virulence gene for *Staphylococcus aureus*, which results in the disruption of the virulence and stops the colonization of this pathogen. CRISPR-Cas9 has also been used to eliminate antimicrobial resistance genes found in plasmids within *S. aureus* and *E. coli*[51]. This could be useful for treating antibiotic-resistant infections by making the pathogens sensitive to antibiotics again. The CRISPR-Cas9 system is usually introduced into pathogenic bacteria by bacteriophages. Recent advances in increasing the specificity and storage capacity of bacteriophages make it realistic to envision the use of genome-editing techniques for clinically treating bacterial infections[52].

Archaea are often difficult to study using traditional genome-editing techniques, but the CRISPR-Cas system offers a promising alternative. Genetically modifying archaea that live in extreme environments is complex because most gene-editing techniques (including the conventional *S. pyogenes* CRISPR-Cas9) cannot work under extreme conditions. In these cases, researchers typically take advantage of the fact that 85% of archaeal genomes contain endogenous CRISPR-Cas systems to repurpose the endogenous CRISPR-Cas system of the archaea of interest as a genome editing technique for that archaea. Archaea genome-editing systems are also of interest because genome editing in some archaeal species has resulted in homologous and non-homologous repair patterns that are very different from those of bacteria or eukaryotes. Given that in some cases homologous repair is the desired result of editing, an option worth exploring is the use of some archaeal proteins involved in the DNA repair mechanisms in other organisms in an effort to favor homologous repair as occurs in the archaea[53]. A further application of genome-editing techniques in archaea is the use of CRISPRi to silence various genes. This has been used to characterize the genes involved in a nitrogen fixation pathway of a species of archaea[54]. Similarly, this technique could be applied to study the characteristic properties of extremophilic archaea and to discover proteins with unique properties.

4.2. Applications of genome editing in fungi and microalgae

Both fungi and microalgae are relevant microorganisms for several industrial processes. Fungi are subdivided into yeasts and filamentous fungi, which include

molds and mushrooms. Among yeasts, *Saccharomyces cerevisiae* stands out for its use in the food industry and as a model organism in research. Although the homologous recombination mechanism of *S. cerevisiae* is so effective that it allows the genome to be modified without using modern editing techniques, the use of CRISPR-Cas has improved the accuracy of the editions and made it possible to modify multiple genomic regions at once. For example, CRISPR-Cas9 has been used to create a *S. cerevisiae* mutant resistant to high temperatures and fermentation inhibitors, which are highly valuable for the alcoholic beverage industries (such as the beer industry) and for industries based on organic acids or glucose fermentation[55].

Genome-editing techniques also facilitate editing in other fungal species that do not have a homologous recombination mechanism as effective as that of *S. cerevisiae*. This is the case of filamentous fungi, which are an important source of active pharmaceutical compounds that include the most widely used antibacterial products (penicillin and cephalosporin), some antifungals, and statins used to lower blood cholesterol levels. Genome-editing techniques make it feasible to modify the metabolism of filamentous fungi of industrial interest to improve the synthesis of these complex bioactive compounds[49]. In addition, they allow the study of the biochemical mechanisms responsible for the pathogenesis of some fungi that infect plants, thus facilitating the development of new antifungal compounds.

Microalgae are used in the industrial production of several natural products, including carotenoids, fatty acids, and pectins. Nonetheless, the most promising application is using microalgae biomass to produce biodiesel. Unlike the use of plants as a source of biomass, microalgae have greater photosynthetic efficiency, grow faster, present better tolerance to biotic and abiotic stresses, while their cultivation does not interfere with food production. Under normal culture conditions, microalgae accumulate both carbohydrate and lipid reserves. For the production of biofuels, they are grown under stress conditions to increase the amount of accumulated lipids (which make up the biofuel) and decrease the amount of carbohydrates, but this also results in reduced growth rates and productivity. Ideally, a microalgae strain would have an enhanced lipid composition without this compromising its growth rate. Genome-editing techniques have been used in an effort to achieve this end, that is, improving the properties of microalgae strains by redirecting the flow of nutrients to lipid production without negatively impacting their growth[49,56].

5. GENOME EDITING IN PLANTS AND GMO FOOD

Genome-editing techniques can accelerate the creation of new plant varieties. Unlike classical techniques based on hybridization or induced mutagenesis, genome editing allows the introduction of modifications aimed at improving a specific characteristic of interest. New plant varieties offer faster growth, higher yields, and better tolerance to both abiotic and biotic stresses such as drought and infections, respectively. Yet, the use of genetically modified organisms (GMOs) in food is controversial and is regulated differently around the world. It should be borne in mind, though, that the use of genetically modified plants is not limited solely to the food industry and that they are also frequently employed in the production of biofuels and other products of industrial interest and in gardening.

To understand the impact of genome-editing techniques on plant modification, this section reviews the evolution of plant variant creation throughout history and the uses of genome editing, including the ecological, ethical, and legal challenges faced.

5.1. Historical perspective

Humans have been manipulating the genome of plants and domesticating them for at least the last 10,000 years[57]. The initial domestication of plants involved the selection of individual plants that presented characteristics of interest. These traits included the better taste or better morphology of seeds and fruits, less toxicity of the edible parts, and greater retention of the seeds in the plants to facilitate their harvest. Through the process of artificial selection, the genetic variants responsible for the traits of interest became increasingly abundant in the domesticated crop population.

A good example of the above is provided by the domestication of maize, a process initiated between six and ten thousand years ago in southern Mexico. Originally, the cereal grain was derived from a primitive variety with numerous lateral branches and cobs of 5-12 grains that fell when ripe. Thanks to the human selection of rare and interesting variants resulting from naturally occurring mutations, the current variety was obtained with its single stem and cobs with dozens of bulky grains, enclosed within a leaf (Figure 7A)[58]. Similarly, the current variety of tomato was derived from a small, tasteless variant; the carrot was originally woody, full of knots, and white; and the current variety of strawberry is a hybrid of a species valued for its taste and another valued for its size[59].

The knowledge about genetics acquired during the early 20th century improved understanding of the selection process. As a result, the expression of traits of interest could be deliberately modified by the crossing of parents, a technique that is still used today to obtain variants of interest (Figure 7B). Because genetic diversity is essential for the breeding process, methods based on induced mutagenesis were developed using chemicals or X-rays to accelerate the frequency of mutations[60]. These processes cause random mutations throughout the genome, so a multi-generation screening and selection process is needed to identify the mutants of interest. The FAO/IAEA Mutant Variety Database records more than 3,300 mutant varieties in more than 230 plant species worldwide that have been created by induced mutagenesis or cross-breeding and artificial selection since 1950[61].

Despite the popularity of the use of induced mutagenesis techniques to create new plant varieties, the extensive process of screening and selection of favorable random mutations makes it a slow, costly process. Hence, the development of genome-engineering techniques in the 1970s aroused great interest because they could target mutations in plants, thus avoiding the selection process. Together with the possibility of regenerating whole plants from cells, the insertion of DNA in the plant genome by biolistics (injection of particles containing genetic information in plant cells) or using *Agrobacterium tumefaciens* made it possible to insert a specific gene of interest in a random position of the plant genome. Today, most genetically engineered varieties have been created using these techniques to insert a gene from another species into the plant genome, thus creating a transgenic organism. A good example of this is the resistance to insects conferred by a gene from the bacterium *Bacillus thuringiensis* (Bt). The genetic character of Bt has been introduced in plants such as corn, soybeans, cotton, and eggplant to prevent insect infections and, thus, reduce the use of chemical pesticides (Figure 7C)[62].

However, the most common methods of transformation using biolistics or *Agrobacterium tumefaciens* randomly introduce transgenic sequences into the plant's genome, which carries the risk of breaking an endogenous gene or altering

its expression. For this reason, the development of site-directed nucleases (SDNs) such as ZFNs, TALENs and especially CRISPR-Cas9 has aroused even greater interest in the creation of new plant varieties. The different ways of using SDNs have been classified into three categories by the European Food Safety Authority (EFSA) in its efforts to define specific regulations for each[63]. Category SDN1 includes the modification of the function of a gene through the non-homologous repair of the double-stranded cut; category SDN2 includes the precise change from one genetic variant to another through homologous recombination with a short fragment of DNA that acts as a template; and category SDN3 includes the insertion at the cut-off point by homologous recombination of a long fragment of DNA that may include more than one gene.

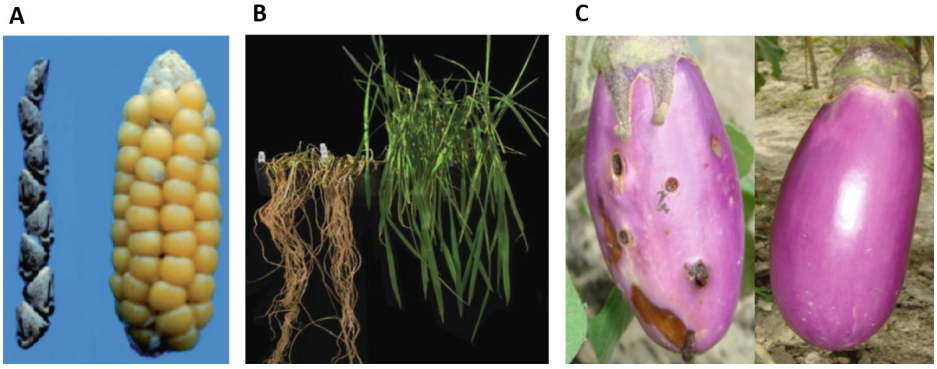

Figure 7. *Plants before and after genetic modification. A) The current variety of maize (right) was obtained from a primitive variety (left) through the human/manual selection of varieties of interest. B) Basic knowledge about genetics allowed the intentional creation of new varieties by crossing varieties of interest, exemplified here with the result of crossing a variety of rice intolerant to immersion with another tolerant variant (right). The intolerant variety before crossing is illustrated on the left. C) Genetically engineered techniques can be used to introduce genes from other species into the genome and confer new properties on plants – for example, introducing a Bacillus thuringiensis (Bt) gene into the eggplant genome prevents moth infection in the transgenic variants (right) and avoids the damage that can be caused in the natural variant (left).*

Each of the three categories of genome editing has technical advantages over earlier techniques. For example, the use of SDN1 allows the expression of specific endogenous genes to be broken or silenced in order to eliminate undesirable traits. In addition, because SDN1 mutations do not incorporate external genetic material, modified plants are not transgenic and are virtually indistinguishable from plants created with induced mutagenesis methods. Therefore, in some countries these products are exempt from being regulated as transgenic products. The obvious advantage over induced mutagenesis is that when employing SDN1 the mutation is directed and, thus, the selection process is unnecessary. This

means, for instance, that a process aimed at improving coffee cultivation which could take 30 years using traditional methods of hybridization can be shortened to just six with modern gene-editing techniques[64]. Similarly, SDN3 modifications direct the insertion of the foreign gene into a specific region of the genome, thus avoiding the alteration of endogenous genetic material that may occur with the random insertion that characterized traditional methods.

5.2. Applications of genome editing in agricultural crops

Most genetically edited plant varieties have been created using CRISPR-Cas9 to introduce point modifications of the SDN1 type[65]. These varieties offer improvements both in their cultivation (e.g. more efficient water use) and in the product obtained in terms of its nutritional properties. Applications are found in areas as diverse as food, drug and biofuel production and gardening[59].

Many varieties improve crop tolerance to biotic and abiotic stresses. Biotic stresses are those caused by the infection of another living organism, such as fungi, bacteria, viruses, or insects. The most common genetic modification to tolerate biotic stress is the introduction of a resistance gene, as exemplified above with plants that incorporate a toxin from *Bacillus thuringiensis* (Bt) to resist insect infections. The applications of this technique are not limited to food production. Indeed, it has also been used to create a variant of chestnut resistant to the chestnut canker (*Cryphonectria parasitica*) which would allow the reintroduction of American chestnut (*Castanea dentata*) to those lands it formerly occupied in the United States before the devastation caused by the arrival of this parasitic fungus[66].

To address abiotic stresses, varieties have been created that better tolerate drought, extreme temperatures, or soils with high salt concentrations, characteristics that could be especially useful in coping with climate change[59]. For instance, CRISPR-Cas9 has been used to alter a maize gene that allows the cereal to keep growing even when water is scarce, and to remove a gene from rice that results in greater tolerance to high salt concentrations. Herbicide-resistant GMOs, whose use is both widespread and controversial, might also be considered a way of coping with abiotic stress. A good example is provided by genetically modified crops that are resistant to glyphosate, which allows the use of this herbicide to prevent the growth of other plants and maximize the growth of the resistant seed of interest[67].

A further application of genome-editing techniques in plants is aimed at obtaining variants with higher yields and production efficiency, that is, plants that grow more using fewer nutrients or plants that produce more or larger fruits. Understanding plant metabolism means modifications can be made aimed at allocating more metabolites to the processes of interest, as well as improving

the efficiency of nitrogen fixation and photosynthesis. However, enhancing these traits with genome editing continues to be challenging because of a lack of understanding of the genomic regulation of plant metabolism, especially in species that do not receive as much research attention as rice and maize. Greater understanding, however, would facilitate the creation of plant varieties that could make the use of biofuels sustainable, both environmentally and economically. For example, biofuel production could be improved by modifying cell wall polymers to facilitate the release of fermentable sugars that are used to form ethanol[68].

Genome editing in plants is not limited to optimizing their cultivation, but also seeks to improve the properties of the final product. Lignocellulosic biomass (i.e. alfalfa) is the main food used in the production of milk and meat. By editing the genome, alfalfa has been modified to create easier-to-digest variants and variants that can reduce the amount of methane produced by animals. The possibilities for improvement are even greater when it comes to human food. The nutritional properties of fruits and vegetables can be improved by using genetic engineering, increasing the quantity of vitamins, antioxidants, micronutrients and essential amino acids that they contain[69]. A good example is that of golden rice, which was modified to produce beta-carotene that becomes vitamin A once consumed. Its use in developing countries could prevent the death of hundreds of thousands of children each year because of a deficit of this vitamin[70].

In the future, transgenic plants could even be used to produce edible vaccines, that is, edible parts of a plant modified to produce a specific component of a pathogen so that when ingested it generates protection against a disease. The rapid distribution of the seeds of these plants and their cultivation for direct human consumption is under serious consideration as a possible solution to the spread of disease in developing countries. A hepatitis B vaccine in potatoes and a coronavirus vaccine in tomatoes are currently being studied[71].

5.3. Ecologic, ethical and legal issues

The commercial use of genetically modified crops has raised ethical, legal, and biosafety concerns, given the possible implications for the lives of present and future individuals as well as for the nonhuman environment. Most of these concerns are related to the use of GM crops; however, recently developed genome-editing techniques, such as CRISPR-Cas9, pose different challenges to those posed by classical transgenic techniques.

While the first genetically modified crops were transgenics incorporating a gene from another species – resulting in a variety that could not easily be created naturally, CRISPR-Cas9 can be used to generate small insertions and deletions at a specific point in the genome (by means of SDN1 modifications). These mutations could occur spontaneously, so the genetically modified variety is

virtually indistinguishable from a natural variety or a variety created by induced mutagenesis. The only difference is that with CRISPR-Cas9 the mutation is generated with high specificity and efficiency, while the other methods introduce random mutations into the genome and a long, expensive phenotypic sorting process is required to select the best variants.

The number of natural spontaneous mutations per generation is relatively high in agricultural crops, which means introducing a single mutation with CRISPR-Cas9 increases the overall mutation rate very little (Figure 8). In contrast, the processes of mutagenesis induced by chemical compounds or X-rays introduce hundreds and thousands of random mutations at various points in the genome. Therefore, from the point of view of the changes made to the DNA, the point mutations introduced by CRISPR-Cas9 are indistinguishable from natural mutations and the variants might even be considered safer than those created by induced mutagenesis, given that a single position in the genome is modified as opposed to hundreds or thousands of random positions. It should be noted that the presence of CRISPR-Cas9 induced mutations outside the target region (that is, off-target mutations) are virtually non-existent, although modified plants are always analyzed to confirm that no off-target mutations can be detected. Thus, we would expect the point mutations introduced using CRISPR-Cas9 to be legally regulated in a similar manner to mutations resulting from hybridization and induced mutagenesis techniques. However, in practice, legislation on genetically modified plants takes completely different approaches and differs markedly between countries.

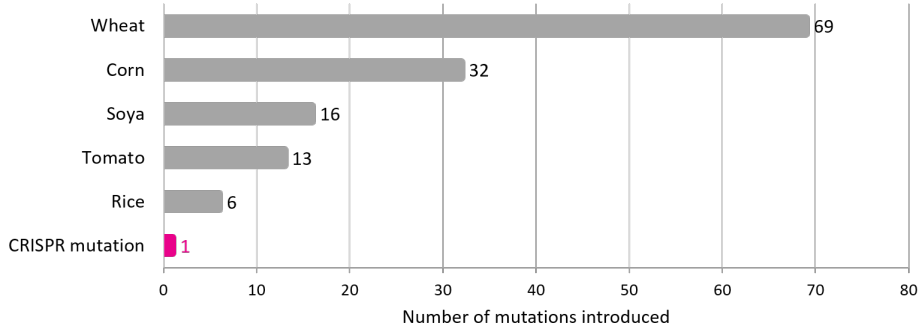

Figure 8. *Estimated number of natural spontaneous mutations in each generation by plant species (gray) compared to the hypothetical single-base change introduced with CRISPR-Cas9 (pink). Adapted from Charpentier et al., 2019*[72].

The legislation in countries such as the United States and Argentina is based on the final product rather than on the process used to obtain it. Because the introduction of small SDN1 mutations with CRISPR-Cas9 results in modifications that are indistinguishable from those that could be obtained with induced or

natural mutagenesis, varieties created with this technique are not considered or regulated as a GMO. In contrast, if the variety obtained contains the small template used in SDN2 modifications or a transgenic element inserted by SDN3, it is considered to be a GMO. Thus, CRISPR-Cas9 SDN1 modifications can be used to create varieties that are not considered GMOs, avoiding the risk analysis that this would otherwise entail and reducing the cost and time of bringing the product to market. While this technical interpretation takes issues of security and efficiency into consideration, it leaves little room for moral, cultural, and socio-economic concerns[73].

By contrast, the legislations of the European Union, the United Kingdom and New Zealand are based on the process used to obtain the product. Thus, these regulations consider that products obtained by SDN1 modifications with CRISPR-Cas9 should be regulated as GMOs, as the process of using these genome-editing techniques for mutagenesis does not occur natural. This means that the use of CRISPR-Cas9 is not as advantageous as in other countries because the GMO approval process delays entry of the product into the market[74]. In the case of the European Union, even after a product has been approved for marketing by the European Council, several countries are likely to ban its use because of public opposition to GMOs. Moreover, the European Union requires manufacturers to label these products as GMOs. An obvious disadvantage of this approach is the difficulty of ensuring compliance with the law, as it is virtually impossible to identify the method used to introduce a mutation that, at the sequence level, is identical to those that could occur naturally[75]. Changes to the existing legislation have recently been proposed to make it product- rather than process-based and, in this way, allow researchers to take advantage of modern genome-editing techniques[76].

6. GENOME EDITING IN ANIMALS

The use of genome-editing techniques in animals opens the door to a wide variety of applications. On the one hand, genome-editing in animals makes it possible to create disease models that, unlike models based on iPSCs or organoids, are whole-body models. On the other hand, as in the case of agricultural crops, genome-editing can be used in livestock to improve nutritional properties or confer resistance to certain diseases. Furthermore, genome-editing in animals paves the way for innovative applications that will require debating the ethics of their use in xenotransplants, the revival of extinct species, or the gene drive, among others.

6.1. Genome editing in *in vivo* disease models

Animal models of disease are essential for understanding the mechanisms of human diseases and for testing drugs to treat them. Unlike *in vitro* models based on stem cells or organoids, *in vivo* animal models facilitate the study of the effects of the disease and/or the drug on the whole organism. This means researchers can investigate the toxicology of drugs and their effects on different organs affected by the recreated diseases.

Genome-editing techniques with nucleases, such as CRISPR-Cas9, have several advantages over traditional targeted mutagenesis methods, including their ability to edit multiple genes at once and to generate genetically edited animal models in a single generation. This not only reduces the cost and time required to create a new disease model, but also helps to comply with the 3Rs principle of animal experimentation (that is, replace, reduce, and refine) by reducing the number of animals required to create the disease model.

The development of new non-human animal models with CRISPR-Cas9 typically involves the injection of Cas9 and one or more gRNA directly into the

animal embryo, or its derivation from embryonic stem cells with the mutation of interest. Alternatively, models can be created with mutations in specific organs or tissues through the systemic injection of genome-editing nucleases into viral vectors with tropism for that particular organ or tissue. Moreover, the versatility of the CRISPR-Cas9 system allows the genetic manipulation of a very wide range of species, facilitating the creation of disease models, in addition to the model organisms in genetics (such as *Caenorhabditis elegans, Drosophila*, zebrafish and mice), in non-traditional organisms such as the red bug (*Pyrrhocoris apterus*), beetles, ants and squid, among others[77].

In this regard, mice stand out as widely used models for biomedical research – especially given the similarity of their genome with that of humans and the fact that they are relatively easy to breed. Genome-editing techniques are especially useful for creating mice models of different types of cancer, a disease caused by mechanisms involving various genetic mutations. The possibility of modifying several genes simultaneously allows the generation of models with tumors of similar complexity to those presented by cancer patients, such as brain tumors[78] or acute myeloid leukemia[79]. These models can be used to identify the genes that are important in regulating cancer and which are responsible for the tumor phenotype. For example, the role of the p53, Lkb1, and Kras genes in lung cancer was identified in a mouse model of lung adenocarcinomas created with CRISPR-Cas9[80].

Depending on the disease being modeled, some organisms are more suitable than others. Germline editing with CRISPR-Cas9 has facilitated the creation of disease models with mammals such as rats, rabbits, pigs, and even nonhuman primates[81]. Pigs, for instance, are more suitable than mice in the study of human cardiovascular disease (e.g. myocardial infarction, dyslipidemia, and electrophysiological disorders) because of their greater similarity to human physiology, anatomy, and genetics. For example, the mutation of fibrillin 1 (FBN1) with ZFNs in the pig germline by nuclear transfer allows models to be created of the Marfan syndrome, responsible for cardiovascular and musculoskeletal disorders, and these models are useful for studying the pathogenesis and the molecular basis of the disease and developing treatments for it[82].

In some cases, it is preferable to use simpler animal models than the mouse, such as the zebrafish (*Danio rerio*) or the nematode *C. elegans*. Both breed faster and take up less space than the mouse, with the added advantage that their embryos develop outside of the mother and are transparent, which makes it easier to study the initial phases of development. *C. elegans* is characterized by being a small, invertebrate animal, so it is not subject to European Directive 2010/63/EU83[83] and, consequently, thousands of *C. elegans* individuals can be used to test the effect of several genes or drugs in pre-clinical trials. In addition, although *C. elegans* has no skeleton or circulatory system, it shares about 20,000

genes with humans, many of which perform similar functions. Therefore, it is a very practical model for studying cellular and molecular processes based on the alteration of specific genes with genome-editing techniques[84]. The zebrafish, on the other hand, is a vertebrate animal with a circulatory system that shares most tissues and organs with humans (except the lungs, prostate, and mammary glands). Because its embryos are transparent and can be observed under a microscope, it allows the study of the processes of angiogenesis and neurological disorders without damaging the embryo. In this context, genomic modification has been used to create zebrafish models of cancer and muscular diseases to understand their mechanisms and to test drugs[85,86].

6.2. Genome editing in livestock

Human population continues to increase in number and it is estimated that it will be necessary to increase food production by 60% to match demand in 2050[87] This creates the need to find effective systems to produce more food while reducing the environmental impact of livestock. Artificial animal breeding has had a major impact on livestock productivity and, today, genome-editing technologies offer the opportunity to produce healthier, more productive, and fitter livestock.

In recent years, several genome-editing strategies have been developed that can facilitate the control of infectious diseases in livestock. A good example of this is the development of pigs resistant to the porcine reproductive and respiratory syndrome virus (PRRSV), the main infectious disease in pigs around the world. Disruption of the CD163 gene using CRISPR-Cas9 has resulted in pigs completely resistant to PRRSV, something that could not have been achieved using traditional transgenic-based gene-editing techniques[88]. Similarly, pigs have been developed that are tolerant to the African swine fever virus[89], bulls that are resistant to tuberculosis[90], in addition to other animals resistant or tolerant to other viral and bacterial infections[91].

Another application of genome editing is the improvement of animal productivity. Disruption of the myostatin gene (MSTN) with targeted nucleases has resulted in pigs, oxen, and sheep with more muscle mass and less fat. However, to date, AquAdvantage salmon is the only genetically modified animal approved for human consumption in the United States and Canada[92]. AquAdvantage salmon contains a modification in the regulation of its growth hormone so as to prevent growth from being interrupted during the cold season. In this way, the fish can grow to market size in just 16 to 18 months rather than three years. Unlike animals with alterations in myostatin expression, genetically modified salmon reaches the same size and presents the same properties as the unmodified variant, which explains its approval for human consumption.

The birth of a calf with a novel genetic modification has recently been reported. The genetic modification was introduced with CRISPR-Cas9 and involves the introduction in the X chromosome of the SRY gene, which is usually found on the Y chromosome determining the male sex of the individual[93]. The genetically modified calves are male despite having two X chromosomes, given that one of the two contains the SRY gene that activates the development of the embryo as a male. More relevantly, 75% of their offspring will be male and just 25% female. The ultimate goal of this modification is to increase the proportion of male calves that are born, as they require 15% less energy than females to increase their muscle weight and they are, therefore, more efficient in their use of food.

Other applications of genome editing in livestock include improvements to food safety and animal welfare. The regulation and possible use of these genetically modified animals is an ethical challenge and clearly requires a broad plural debate. A good example of this is the introduction of a variant into the ox genome that prevents the formation of horns[94], thereby avoiding the painful removal of horns from the young. However, in this particular case, the insertion of plasmid DNA into the genome of oxen was also detected, emphasizing the importance of defining the risks and consequences of the use of genome-editing techniques.

6.3. Future applications of genome editing in animals

In addition to the development of animal disease models and the improvement of livestock properties, the application of genome-editing techniques in animals opens the door to novel uses that inevitably generate ethical, social, economic, and regulatory debates. This section discusses three of these applications: improved xenotransplantation, species de-extinction, and the gene drive.

Xenotransplantation is the process of transplanting cells, tissues, or organs from one species to another and, recently, research in this area has gained popularity due to the progressive decline in the availability of human donors[95]. Pigs are considered the best candidates as organ donors to humans because of their similar size and physiology along with their high availability. However, there are various risks related to immunocompatibility and the transmission of porcine microorganisms and viruses to the human receptor. Genome-editing techniques, especially CRISPR-Cas9, offer the opportunity to genetically modify pigs for organ donation to avoid or eliminate some of these risks. An example of one such risk is the transmission of porcine endogenous retroviruses (PERVs), that is, retroviruses integrated into the pig genome that could be transmitted to humans through xenotransplantation. CRISPR-Cas9 has been used to introduce 62 modifications into specific regions of the pig genome to inactivate all PERVs

in the genome and eliminate their risk of transmission to human cells[96,97]. Pre-clinical trials are currently underway to assess the safety and effectiveness of the xenotransplantation of genetically modified pig organs in non-human primates[98], while work has begun on drafting legislation to ensure the safety of future clinical trials and to address the ethical implications of this practice.

De-extinction is the process of recreating or reviving a previously extinct species. A good example is provided by the Pyrenean wild goat. In 1999, just one living specimen of the species remained alive in the Ordesa National Park in the Pyrenees. It was captured, an ear sample extracted and released again, only for it to be found dead the following year. In 2003, the tissue sample from this last specimen was used to try to clone and de-extinct this subspecies. To do so, the nucleus of a somatic cell in the tissue sample was transferred to the egg of a domestic goat. Despite implanting several embryos, just one individual was born, but the animal died 7 minutes postpartum from a respiratory problem. However, the goat was genetically identical to the last Pyrenean goat specimen and so its birth is usually considered the first de-extinction[99].

Work is currently underway to de-extinct the woolly mammoth from frozen remnants of its DNA. Unlike the Pyrenean goat, the amount of mammoth DNA preserved is insufficient to transfer a cell nucleus to an egg of a similar species and clone the species directly. This means gene-editing techniques will play a key role in the de-extinction of the mammoth, as they should allow the introduction of mammoth genes into the current elephant genome to reproduce some of the characteristics of the woolly mammoth, such as its thick fur and cold-adapted hemoglobin[100]. Although de-extinction work involves significant advances in DNA editing technologies and could serve to improve ecosystems that humans have destroyed, it is often criticized for receiving funding that might otherwise be allocated to conservation programs of current species.

Gene drive is another application of genome-editing techniques that could have significant repercussions. Gene drive is a technology that propagates an altered genetic character through a wild population at a higher rate than by conventional inheritance. In fact, it is a fundamental process so that genomic changes introduced in one or just a few individuals can spread through populations. The technique is based on using CRISPR-Cas9 so that 100% of the offspring of a cross between a modified and a wild organism inherit the altered character, as opposed to the 50% that would be obtained in a conventional cross. Thus, starting with the introduction of a small number of genetically modified organisms containing the gene drive system, the altered character spreads through the wild population as it crosses with the modified one.

To ensure that 100% of the offspring have the altered character, the organism with the gene drive contains CRISPR-Cas9 in its genome. When it mates with a wild organism, the offspring acquire a copy of the gene drive system and a

non-modified copy of the wild parent. During early development, the copy of CRISPR-Cas9 integrated into the gene drive system cuts the non-modified copy. This cut is repaired using the gene drive system as a template, resulting in offspring that have two copies of the genetically modified variant with the gene drive. As a result, the offspring will pass the gene drive system to 100% of the next generation (Figure 9).

The genetic impulse technique has the potential to alter entire populations and, therefore, modify entire ecosystems. One of the risks is that the altered traits spread beyond the target population[101]; however, recently developed techniques make it possible to control the genetic impulse through transmission efficiencies of less than 100% and the progressive introduction of modified individuals into the wild population. Proposals have been made to use gene drive to reduce or eliminate insect-borne diseases (such as malaria and dengue), to control invasive species, and to reverse insecticide resistance in pests. To date, gene drive testing has been performed in controlled and isolated populations, and the first tests of the effects of the release of genetically modified but sterile mosquitoes on the outside environment are being conducted[102]. It will be a few years before we see the first applications of gene drive in the wild and can determine whether the results are as promising as they seem at the outset. In the meantime, the effects of gene drive on ecosystems and the evolutionary dynamics of the species affected need to be studied in depth. Indeed, gene drive is a technique that might not only modify certain individuals but direct the evolution of a whole population. As such, it represents a massive step in genome editing and has enormous implications.

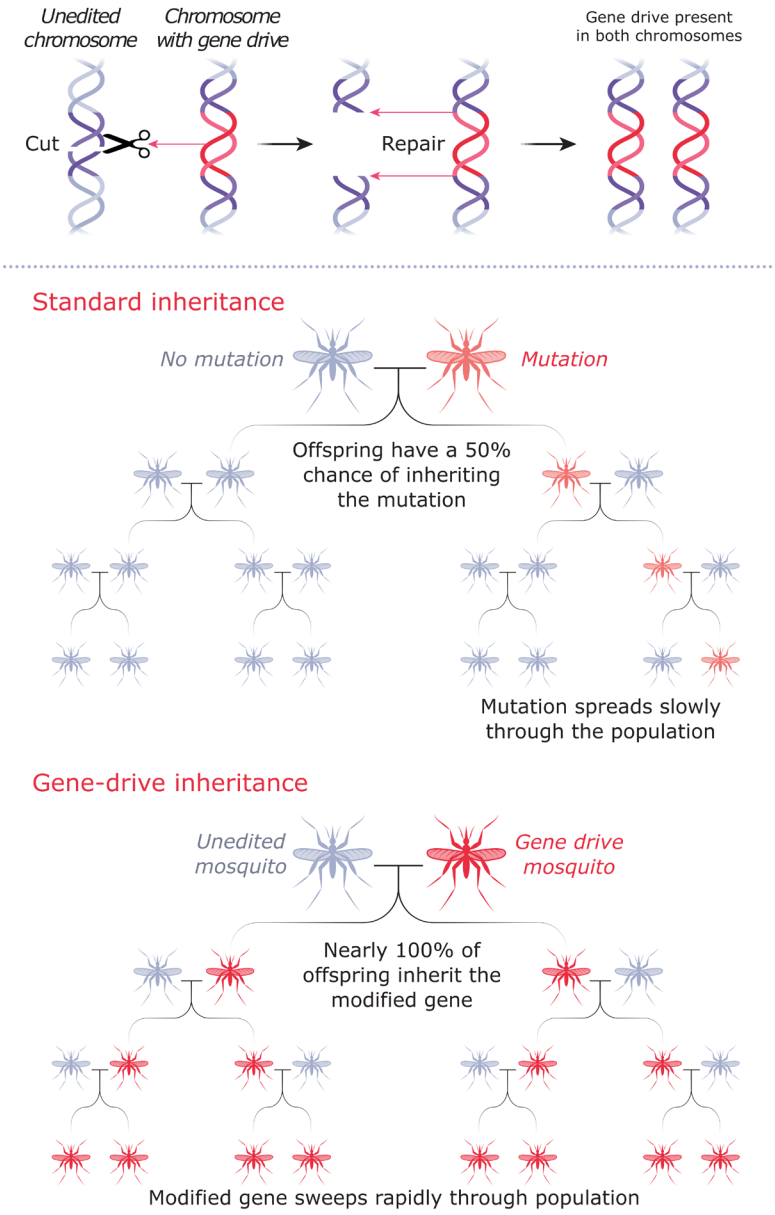

FIGURE 9. *The gene drive mechanism. The crossing of a mosquito with a gene drive chromosome and a wild mosquito results in offspring with a copy of the gene drive system and a non-modified copy. In the early stages of development, the CRISPR-Cas9 system integrated into the drive cuts the non-modified copy and, subsequently, this cut is repaired using the gene drive as a template. As a result, 100% of the offspring pass on the gene drive to the next generation. In standard inheritance, mutated characters propagate slowly as only 50% of offspring inherit them whereas, in gene drive inheritance, modified characters spread rapidly because 100% of offspring inherit the modified gene. Adapted from Scudellari, 2019*[102].

7. HUMAN GENOME EDITING

The rapid development of genome-editing techniques raises the possibility of altering human traits in a very precise fashion, opening the door to modifications of human biology that hitherto were quite inconceivable. The therapeutic potential of these techniques is enormous, ranging in scope from correcting or treating genetic diseases to altering certain cellular receptors to prevent, for example, infections such as HIV. But, in addition to their therapeutic applications, genome-editing techniques will enable applications that require ethical debate and strict regulation, the case, for example, of designer babies and the enhancement of human qualities.

The consequences of editing, moreover, vary greatly depending on the choice of the genomic region and the cells to be modified. The editing of cells in the germline (which will form the gametes) and that of cells in the somatic line are relatively similar processes from a technical point of view but their use has radically different implications. Changes to the genome of a somatic cell propagate solely to the offspring of that cell, resulting in mosaic tissues made up of some edited and some unedited cells. In this case, the persistence of these modifications is limited to the maximum life span of the individual, as they are not passed on to offspring. In contrast, genome modification of a germ cell has the potential to create an individual in which most cells will present this modification, including the germ cells, so that the manipulation will be passed on to offspring.

This section explores the benefits and challenges of the therapeutic editing of human cells, distinguishing between somatic and germline cells, and discusses the risks associated with improving human traits.

7.1. Somatic genome editing

Gene therapy involves transferring DNA to the cells of a patient to correct a defective gene or its effects so as to treat diseases that cannot be cured with conventional drugs. The first clinical trials in gene therapy were conducted in the 1970s, but it was not until 2017 that the FDA (followed in 2018 by the European Medicines Agency) approved the first gene therapy, voretigene neparvovec. This therapy uses a viral vector to deliver a correct copy of the RPE65 gene to retinal cells, repairing mutations that cause Leber's congenital amaurosis (LCA) and improving the vision of those affected. In fact, the first gene therapies developed were based on the use of vectors to deliver correct genomic fragments to replace mutated fragments that cause disease[103,104]. Advances in genome-editing techniques have ushered in a new type of gene therapy based on the use of nucleases, such as CRISPR-Cas, ZFNs, and TALENs, to directly repair the mutation that causes the disease or its effects. This section focuses on these more recent therapies, for which several clinical trials are underway, some of them expected to hit the market in the next 5 years.

Most current clinical trials are focused on ex vivo gene therapies, a process that involves the genetic modification of cells outside the body to produce therapeutic factors, followed by the transplantation of the patient's edited cells. *Ex vivo* editing has certain advantages over *in vivo* editing, including the fact that it facilitates the entry of genome-editing enzymes into the target cells and it prevents immune responses that would compromise the safety of the treatment. Thus, it is hardly surprising that the first clinical trials of genome editing were ex vivo therapies designed to treat blood diseases such as sickle cell anemia and beta-thalassemia. The treatment strategy is to obtain hematopoietic stem cells from the patient, edit them with ZFNs or CRISPR-Cas9 to produce the fetal form of hemoglobin (the production of which normally stops during the first months of life), and reintroduce them to the patient so that the fetal form of hemoglobin assumes the function of adult hemoglobin affected by sickle cell anemia or beta-thalassemia[105]. Another type of *ex vivo* therapy for which several clinical trials are underway is the editing of T lymphocytes, given their accessibility and potential for use as cancer immunotherapy. Specifically, CRISPR-Cas9 is used to enhance the characteristics of modified T lymphocytes expressing a chimeric antigen receptor (CAR-T cells) that recognizes a carcinogenic marker and directs tumor clearance (Figure 10)[106].

In the case of *in vivo* gene-editing therapies, nucleases that edit DNA include the drug itself. The current application of these therapies is largely limited by the methods of delivering the DNA editing enzymes to the somatic cells to be edited. This means ongoing clinical trials are limited to diseases that can be treated by editing easily accessible tissues. Eye diseases, for example, are good candidates for multiple reasons, including the ease of access to the retina; the immune

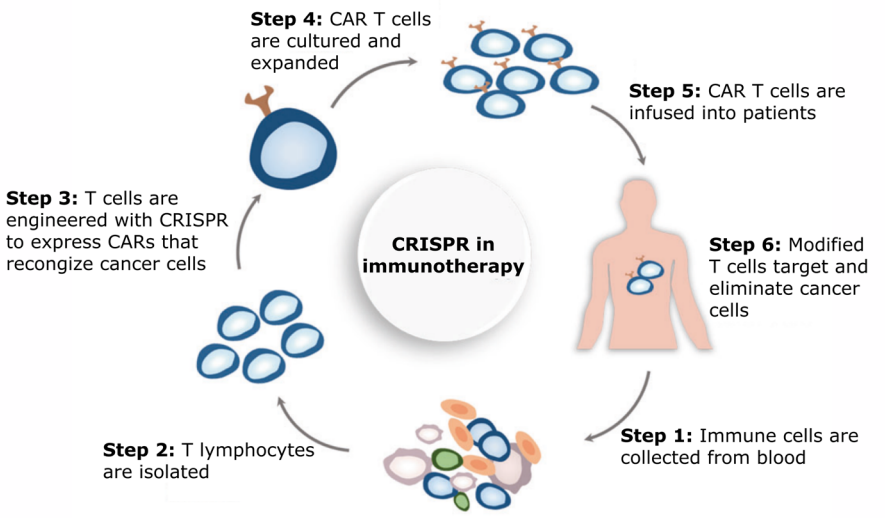

Figure 10. *Ex vivo gene therapy based on CAR-T lymphocytes modified using CRISPR-Cas9. Adapted from Li et al., 2020*[105].

privilege of the eye, which reduces the risk of immune response to vectors or external genes; the poor circulation between the eye and the rest of the body, which reduces the risk of editing other body tissues; and, the low replacement of edited cells, which improves the likelihood that the treatment persists longer. The most advanced in vivo genome editing clinical trial today is precisely to treat LCA by editing retinal cells with CRISPR-Cas9[107].

In the years to come, we can expect to see the first clinical trials based on cutting-edge genome-editing techniques, such as base editing and prime editing, and which have the potential to correct up to 90% of the 75,000 human pathogenic variants described in ClinVar[24]. However, we should not expect the current framework for monogenic diseases to be exceeded. Moreover, until there is an improvement in the delivery systems of DNA editing enzymes to the somatic cells to be edited, gene therapy is expected to be limited to diseases that can be treated *ex vivo* or from externally accessible tissues[108]. These limitations explain why there is so much interest in editing the human germline.

7.2. Germline genome editing

While treatments based on the edition of the somatic line are faced primarily by technical problems, germline editing generates a much more complex ethical debate. Germline editing involves the genetic modification of either the gametes

used for *in vitro* fertilization (IVF) or a zygote or embryo in the early stages of development. If this embryo is implanted, the individual born will have this edition in most, if not all, of the tissues of their body, including the germ cells that then transmit the modification to the individual's offspring. Thus, while the consequences of somatic editing are limited to the lifetime of one individual, those of germline editing are transmitted and passed down from generation to generation, which is why special attention must be paid to their possible negative consequences and technical, social, ethical and economic implications.

The editing of human embryos for use in biomedical research has shown that the technical limitations of current genome-editing techniques are considerable and that germline editing is not currently sufficiently safe to be used as a treatment[109]. This means that editing the germline for any use other than research is prohibited worldwide. Technical limitations include the fact that the outcomes of edits performed using nucleases (such as CRISPR-Cas9) are not always as desired, while there is a certain risk of editing outside the region of interest (off-target). In addition, because the full efficiency of these techniques cannot be guaranteed, there is a risk that some cells in the embryo remain unchanged and the resulting individual is a mosaic in which not all cells have the mutation that cures the disease (Figure 11)[110]. Editing gametes as opposed to zygotes or embryos would avoid some of these limitations, as all the cells in the embryo then formed would be identical (avoiding mosaicism) and this would allow the embryos with the correct mutation. However, gamete gene-editing techniques are still under development.

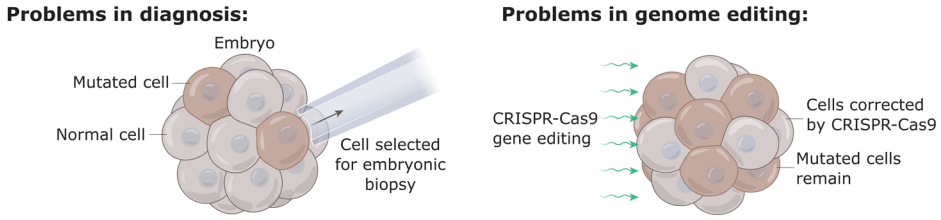

Figure 11. *Mosaicism in genetically edited embryos. Mosaic embryos have cells with different mutations, which can lead to misdiagnosis (left). When embryos are edited with CRISPR-Cas9, because the efficiency of the process is not 100%, a mosaic is created as some cells have not been edited. Adapted from Ledford, 2019*[110].

Before germline editing is deemed sufficiently effective and safe, there needs to be a broad plural discussion of its social and ethical impacts, limiting its applications to cases where it is considered strictly necessary. The most likely area for its application would be for correcting mutations that parents want to avoid passing on to offspring. However, even here, alternatives exist, including

adoption or, if the parents want to have biologically related offspring, the use of preimplantation diagnosis to select embryos that will not inherit the mutation. Thus, eventually, germline editing seems likely to be limited to those cases where the probability of obtaining an embryo without the mutation is very low, since it can be argued that it is better to correct embryos than to produce lots and then discard the majority simply to find one without the mutation[111]. Another instance in which germinal editing might be adopted is in the treatment of diseases for which somatic editing has not proved entirely effective. Examples include cystic fibrosis and muscular dystrophy, given that the somatic therapies currently being studied are only able to act on some of the tissues affected by these diseases, whereas edition of the germline would cure the disease in all tissues[112].

The first attempt to edit the human germline was reported in November 2018 and aimed to create humans resistant to HIV infection. To do so, a team led by He Jiankui, at that time a member of the Southern University of Science and Technology in Shenzhen (China), attempted to recreate a mutation in the CCR5 gene that is found naturally in some people and is associated with resistance to HIV. The goal was to create modified individuals who would be resistant to the virus, although a separate study, unrelated to the experiment and published a few months later, concluded that the deletion introduced could shorten the lives of individuals. This underscores a critical feature of gene therapies: even when the desired genetic change has been introduced, it is difficult to ensure that the alteration will have no unexpected consequences for other biological processes. Therefore, it is vital that our understanding of the chosen gene and the effects of the introduced mutation are perfect before proceeding with the therapy.

This attempt to edit the human germline was controversial for several reasons. First, the laws of many countries, including China, prohibit germline editing as a therapy. Yet, the fact that the experiment could be carried out demonstrates the complexity of the strict regulation of the use of genome-editing techniques and the possible risks of their being misused. Second, a number of scientists have pointed out that alternative techniques can be employed to achieve the goal of this mutation and that, therefore, the use of genome editing as a last resort was not justified in this instance. Finally, the experiment has also been criticized for the lack of transparency in the process and for the failure to initiate a public debate on the ethics of the therapy before starting it[113]. As a direct result of the ensuing scandal, scientists proposed a global moratorium on germline genome editing until the many ethical and technical questions had been clarified, but this moratorium has yet to be put into effect. The ethical implications of germline genome editing are discussed in section 8 below.

7.3. Human genetic enhancement

Genome-editing therapies aim to treat a genetic condition, either by changing the genome to improve the symptomology or by reversing the pathological effects of the disease. In contrast, genetic enhancement involves "improving" the individual or their offspring in order to provide qualities that are considered beneficial but not originally encoded in the genome. This change in concept from treating to improving raises a number of important ethical questions.

First, the distinction between which conditions can be considered a disease and which not is sometimes unclear and may depend on the context. Human characteristics are the result of many genetic variants and cover a diverse spectrum, which means that what might be deemed normal for one person may be deemed abnormal for another. For example, there can be no disagreement that Tay-Sachs disease is not normal and should be classified as a disease because of its undesirable consequences; but, the distinction is less clear, for instance, in the case of genetic deafness. Although not consistent with the range of abilities typically associated with the human species, this characteristic includes the individual in a community of people also affected by genetic deafness, some of whom reject the idea that deafness should be cured or treated. Thus, a deaf couple might prefer to have a deaf child that shares with them the experience of living with this condition[114]. The case of color blindness is perhaps even clearer, given that it is a condition that should not be considered an anomaly, affecting only a minority (roughly 7% of men and 0.5% of women). It should therefore be borne in mind that the concept of disease is neither objective nor neutral, but a social outcome that is likely to be influenced by factors not strictly related to the biology of the condition.

Second, the line between therapy and improvement is, in many cases, blurred. Improvement can be defined as the alteration of human abilities beyond the typical level or the normal range of functioning for the species, or as any non-therapeutic intervention to change a human trait. Treatment, on the other hand, aims to restore normal function and is considered improvement if it goes further than that. While this distinction may seem simple, it can give rise to confusion. For example, it is unclear whether genome editing should be considered therapy or improvement if it is used to introduce a natural variant present in a small part of the population, say in order to lower blood cholesterol levels. It is also unclear whether correcting the BRCA gene mutation to avoid a high risk of cancer should be considered treatment because of its preventive nature. It may even, on occasions, be impossible to separate treatment from improvement. Imagine, for example, an individual that has been treated to restore normal muscle function, but the therapy goes a little further and more efficient results are obtained. In cases where the efficiency is higher than expected, an improvement would unintentionally have been introduced.

The use of growth hormone, while not part of the field of genome editing, illustrates the difficulty of drawing a clear distinction between prevention, therapy, and improvement. Initially, growth hormone intake was restricted to those with lower than normal hormone levels. Later, it was extended to people shorter than the 1st percentile in height for their age, regardless of the natural levels of the hormone in the individual. Elsewhere, others with normal height were given growth hormone to improve their strength and height above the average. The fact that growth hormone is usually given to children who are too young to make decisions for themselves adds to the complexity of the matter. Currently, the use of growth hormone is strictly regulated and is only allowed if hormone levels are lower than normal or if height is significantly lower than normal. Yet, its use still involves the making of complex decisions, especially given the lack of consensus that exists as to whether short stature is even a condition that requires treatment and the possible adverse effects of growth hormone treatment[112].

In the distant future, genome editing may serve to alter complex characters, such as intelligence, facial traits and athletic ability, whose biological and genetic bases have yet to be understood. Meanwhile, the genetic enhancement debate remains an active topic of discussion in bioethics. Some philosophers, like Michael Sandel, are oppose to the enhancement of human embryos, arguing that it would increase inequalities between those who have access to the techniques and those who do not, adding biological inequalities to the social ones that exist today. In addition, they argue it would put too much pressure on those who have access to them to opt for improvement instead of the natural option[115]. Others, such as Julian Savulescu, justify the genetic enhancement of embryos by comparing it to the use of drugs or participation in extracurricular activities that improve children's cognitive and sporting abilities, and some even claim that parents have the moral obligation to provide the best possible life for their descendants[116]. Whatever the case, it is crucial that a plural diverse debate be held about using genome-editing techniques to improve human qualities before it is too late[117].

8. ECONOMIC, LEGAL, ETHICAL, AND SOCIAL ISSUES

Applications of genome-editing techniques today extend across all domains of the life sciences and their transformative potential impacts society at the economic, ethical, and cultural levels. Indeed, this impact can be expected to grow further as technical issues are resolved and new applications based on these techniques are developed. It is, therefore, essential that we start deliberating the impact of these techniques now so as to ensure that the transformation of society is as beneficial to as many people as possible and that the risks that might arise from using them can be avoided. As the legal regulation of gene-editing techniques in food and agriculture has been discussed above, this section focuses specifically on the economic impact of these techniques, the CRISPR-Cas9 patent dispute, the social impact of germline editing, and their public perception.

8.1. CRISPR-Cas9 PATENT LANDSCAPE

Genome-editing techniques have been adopted in almost all industries involving biological systems. In addition, their clinical use for gene therapy promises to cure a number of severe diseases very soon. Along with other advances currently being made in the field of biotechnology, a bio-revolution is expected in the very near future that will transform agriculture and health and incorporate living organisms in the creation of materials and energy. Industries working in these fields look set to receive 30% of total private investment in research and development[118]. While in the next decade most of this investment will be devoted to applying the biological knowledge obtained by omics via data analysis and artificial intelligence techniques, it is estimated that over the next 20 years the importance of genome-editing techniques will grow from capturing 30% of investments to 70%[118]. All this is likely to be accompanied

by an increase in the total annual economic impact of the bio-industry, which in a period of 20 to 30 years could grow from 1 to 4 trillion (1-4 x 10^{12}) US dollars (Figure 12).

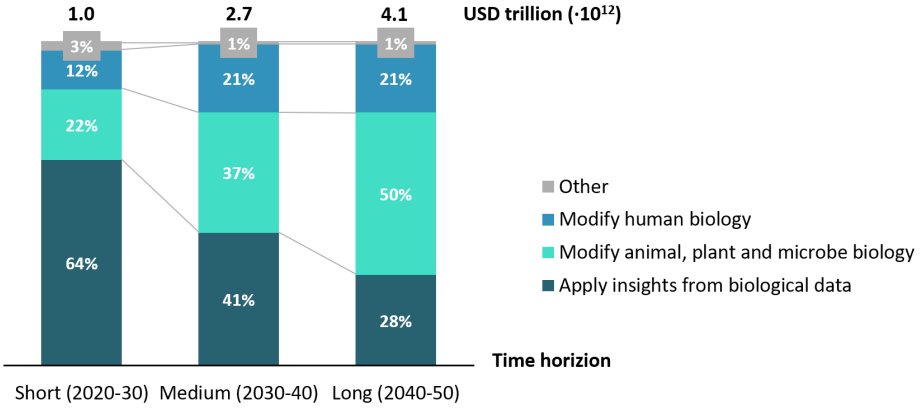

FIGURE 12. *Distribution of the economic impact of biological applications depending on the technological platform. The total impact of the life science industries is set to grow from 1 to 4 trillion US dollars. Applications based on knowledge provided by biological data represent most of the economic impact created in the short term, but applications based on genome editing are likely to go from representing 30% of the total economic impact to 70% within a period of 20 to 30 years. Adapted from McKinsey Global Institute, 2020*[118].

Given the economic impact that genome-editing techniques may have in the coming years, patents that ensure the exclusivity of their commercial use are of paramount importance. Yet, procedures for patenting biological products and techniques differ around the world. For example, in the European Union, genetic sequences can be patented even if they are natural, as long as their use and industrial application are specified. However, in the United States, genetic sequences identical to those occurring in nature cannot be patented, as their discovery is not considered to be novel given that they already exist in nature.

Thus, in the case of the CRISPR-Cas9 system, in Europe it was possible to patent the gene encoding the Cas9 enzyme as found in nature as long as its industrial application was also specified. In contrast, in the United States, only methods and components specifically modified from their natural form in developing a certain application can be patented. The dispute surrounding the first CRISPR-Cas9 patents in the United States broke out in 2012 between the two main players in its adaptation as a genome-editing technique. On the one hand, the patent application made by the University of California Berkeley, the University of Vienna and the scientist Emmanuelle Charpentier (a group often referred to as "CVC") is based on the work of Jinek *et al.* published in

August 2012[10] and claims to patent the fusion between the two RNA fragments forming the gRNA and the use of CRISPR-Cas9 to edit *in vitro* DNA sequences. On the other hand, the patent of the Broad Institute, MIT and the scientist Feng Zhang (a group often referred to as "Broad") is based on the work of Cong *et al.* published in February 2013[12] and claims to patent modifications to the CRISPR-Cas9 system that allow genome editing in eukaryotes.

Although CVC's patent application was made in May 2012 and Broad's in December 2012, Broad's patent was granted first (April 2014) because the group had requested a fast-track examination of the patent. In the United States, when two groups of inventors lay claim to overlapping inventions, priority is given to the group that can demonstrate it was the first to put the invention into practice. Thus, in 2015, CVC requested an interference be declared between its patent and that of Broad, on the grounds that that the edition of eukaryotic cells was an obvious extension of its work that simply demonstrated the *in vitro* edition of purified DNA and that, in addition, CVC had actually proposed the application of CRISPR-Cas9 gene editing in eukaryotes before Broad. The 2017 interference resolution, in fact, determined that the claims of the two groups referred to two distinct inventions (the CVC patent referring to the creation of the gRNA and the Broad patent referring to the adaptation of CRISPR-Cas9 to edit eukaryote cells) and that, therefore, the adaptation of Cas9 for use in eukaryotic cells was not obvious from the work reported by CVC. CVC then brought fresh claims that resulted in a second interference being declared. This time round, the Appeal Board focused on determining which group had the earliest experimental evidence that CRISPR-Cas9 worked in eukaryotic cells. On September 10, 2020, the board gave priority to the Broad patent because the evidence indicated that they had been first. However, CVC still has the opportunity to present further evidence to overturn that decision. For the time being, however, it is expected that Broad will be able to maintain the eukaryotic cell editing patent for CRISPR-Cas9, and that CVC will have a less specific patent for the use of CRISPR-Cas9 *in vitro*[119].

When all is said and done, the practical importance of the decision regarding these patents is likely to be relatively small. Indeed, innovations related to CRISPR-Cas9 have diversified into altered compositions and new uses that have been patented separately, resulting in a highly complex CRISPR patent landscape, and a good number of commercial applications of genome-editing techniques depend on patents other than those in dispute between Broad and CVC. What's more, the CRISPR-related patent landscape is markedly different in Europe (where CVC has priority over the original patent) and in other regions of the world.

Ultimately, access to CRISPR-related genome-editing technologies depends on the patent system, but the idea of patenting inventions developed in

universities using public funding (as was the case of CRISPR-Cas9) has raised doubts regarding its legitimacy. Moreover, in some cases these patents form the basis of biotechnological inventions used in products and services that are beneficial to society. The challenge seems to be striking the right balance between providing sufficient freedom and transparency to promote the advancement of scientific research while maintaining some degree of control to encourage private innovation and business development. The goal of the university's patent program should not be to maximize profits but rather to integrate its innovations within its broader social and economic missions.

In practice, this translates into a need for different regulations depending on the uses to which a technique is put. In the case of academic and non-profit research, the institutions that created a technology often share biological materials for free through Addgene, a non-profit organization. However, commercial and private research uses are regulated by patents. Broad has granted exclusive use licenses for therapeutic applications to Editas and other spin-offs created by the institute itself. Similarly, CVC has granted exclusive use licenses to its spin-off, Caribou Biosciences. These spin-offs, in turn, provide sub-licenses for specific applications to several companies not associated with the university. The fact that spin-offs with exclusive licenses granted by the university not only provide sub-licenses to other companies but at the same time seek to develop new products based on licensed technologies could limit access to these technologies[120]. After this system was criticized for delaying therapies, the universities began providing non-exclusive licenses directly to other companies (without using another company as an intermediary) in order to accelerate innovation in this sector.

8.2. Social and ethical issues of human genome editing

The implications of genome editing in humans vary greatly depending on the purpose sought and the cell being edited. Three quite distinct possibilities need to be distinguished. First, there is genome editing in somatic cells, which is being regularly used in both research and therapy. Second, we have editing in human embryos or germ cells, which has been approved for research in embryos left over from *in vitro* fertilization, albeit with strict restrictions regarding the number of days of embryonic development allowed. However, its use in therapy is not permitted for technical reasons and social concerns. Despite these restrictions, in November 2018, He Jiankui announced the birth of the first CRISPR-Cas9 genetically modified babies in a controversial experiment that was to have legal consequences for malpractice. Third, there is the possibility of using genome-editing techniques to improve the human species, which in the future could mean it is feasible to alter physical traits and even such qualities as intelligence. While somatic editing raises no more ethical issues than those concerning

unequal access to these therapies, germline editing for therapy and human genetic enhancement give rise, as we discuss below, to many ethical concerns.

One of the challenges of germline editing is striking a balance between the benefits that accrue at the individual level and the risks generated at the social level. This means we need to estimate the benefits that these techniques would represent to the parents who opt for their use and to their offspring and the risks both they and society run in applying them. Such an estimation is far from straightforward given that the individual consequences are more immediate and specific, while the public perception of the social and cultural benefits and risks are much more diffuse and open to philosophical debate.

At the individual level, the risks are largely technical, and include such outcomes as mosaicism and the uncertainty of the possible consequences of the genomic variants introduced. The latter risk, however, is less important in therapeutic genome editing, since the technique usually restores normal function by introducing genomic variants naturally present in the population and the consequences of such, therefore, are known or can be studied. On the other hand, when it comes to the enhancement of human qualities, genomic variants that do not exist naturally might be introduced and this could lead to unexpected consequences for the whole organism. Here, we are dealing with complex traits and their genetic basis is not sufficiently understood. It might be the case that, owing to the complexity of human traits, improvements are associated with a risk: For instance, how feasible would it be to introduce a genome modification that raises a person's intellectual coefficient by 30 points but which gives that individual a 20% greater risk of suffering a heart attack? And how should we proceed with a mutation that can increase muscle mass, but which in 15% of cases results in severe impairment of cognitive function?[117]. In such cases, parental decisions to accept or reject the treatment would be decidedly fraught with complexity. Indeed, obtaining the consent of genetically modified offspring will also be problematic, as germline modification would require long-term clinical trials affecting multiple generations and would require the consent of all of them (many still unborn) in order to move forward.

At the social level, the biggest challenges concern the inevitably limited nature of access to processes of genetic treatment or improvement and the inequalities this would entail, in addition to concerns about the morality of the process itself. Initially, at least, germline editing will have an enormous cost and will only be available in certain regions of the world. This means it seems likely to add to the social inequalities that currently result in unequal access to vaccines and good nutrition. Others claim that while access would initially be restricted, this is necessary to force prices down until they reach a point where they are affordable for all. Their argument is that this would, in fact, lead to a more egalitarian society by providing treatment for those who have traits that place them and

their descendants at a disadvantage. It is even argued that it would benefit public health by decreasing the prevalence of serious diseases like Huntington's disease, and that there are strong moral reasons for modifying and even, in some cases, of enhancing the human genome[121].

The social consequences of genome editing, however, could extend much further. Although limited to preventing serious illness or disability, the use of the techniques raises concerns that voluntary individual decisions might lead to social changes in terms of the acceptance of less severe disabilities. Some members of the disability community claim that prevention (whether by means of prenatal diagnosis or genome editing) seems to suggest that people with disabilities are a problem and that avoiding disabilities is a priority of the health system, which exaggerates the difficulties that some disabilities constitute. Other members argue that if these techniques are used to prevent disabilities, policies that facilitate the inclusion and accessibility of disabled people will lose support as the number of people with disabilities falls.

Another stance taken against germline editing is that which expresses a preference for a "natural" human genome, typically associated with the opinion that modifying genes is tantamount to "playing God". This latter view stems from the belief that humans do not have the divine omniscience needed to make changes to the genome that are both safe and beneficial. This argument rests on the conviction that natural alterations and evolution are less problematic than human intervention, although they can both modify genes: there is a "sacred" view of the genome, as if it were something superior to humanity itself. Yet, it might be argued that natural alterations are random while human alterations are usually limited to a region whose safety has been studied; thus, the former have greater potential for causing unintended consequences. Moreover, the human genome is not entirely "human", given that it includes DNA from other species (including many viruses and a small proportion of Neanderthals) and, as it is constantly changing, each genome is unique and not shared by the entire of humanity (see the summary "Natural dynamics of evolving genomes" at the end of this report).

Religions also adopt different stances on genome editing. For Christians, the degree to which human beings should be allowed to intervene in nature is an active topic of debate; whereas, Jews see an explicit obligation to build a world that benefits people, which means for them genomic improvements are seen as an opportunity to collaborate with God, rather than an interference with creation. Similarly, Muslims and Buddhists view genome editing as another invention that reduces the suffering caused by disease[112].

Applications of genome editing are open to the "slippery slope" argument, that is, once we have started out on the path it is difficult to know when to stop. Thus, initially, only therapeutic corrections would be accepted but, as these

applications gain acceptance, the transition to the next phase is more readily facilitated and so on until human improvement is normalized[122]. In contrast, others claim that banning techniques based on the "slippery slope" argument is a fallacy, as the path of progressive acceptance is uncertain and insufficient to justify impeding the benefits of the early stages and, moreover, subsequent stages can be averted by introducing strict measures. Furthermore, progressing to other stages will be the responsibility of future generations, who may well have different conceptions to those we hold today.

Taking into consideration the social consequences of genome-editing techniques and the state of the art, the Observatory of Bioethics and Law at the University of Barcelona published a document in 2016 that recommends adopting a gradualist position based on the principle of caution[123]. Thus, it advocates that "in the acceptance of the techniques of genome editing it is necessary to proceed by steps: to allow their use in basic research, to approve therapeutic use in somatic cells, to evaluate the possibility of approving germline therapy in certain cases and to stop their use for human enhancement". More specifically, the report stresses the importance of halting applications for enhancement while waiting for data from the previous two phases and not before conducting a serious deliberation on the risks and benefits based on evidence from these same phases. In addressing the "slippery slope" argument, the report stresses that appeals to this to stop the development of genome editing "carries with it the risk of restricting scientific progress and access to the benefits that these technologies may offer in the future".

Finally, the report stresses the importance of "involving the media and the public in an inclusive, forward-looking and informed social debate". This debate should be public and include citizens, scientists (including research funders and biotechnology firms), public policy makers and the media. The latter should exercise due care in reporting scientific advances to avoid alarmism and exaggerated expectations about their benefits. Scientists, on the other hand, need to pay special attention to the social repercussions of their advances and work to mitigate the risks involved. They are also responsible for involving the public in their research as much as they can and are ultimately responsible for disseminating their results in a rigorous, clear, and understandable manner.

9. BIBLIOGRAPHY

1. Capecchi, M. R. Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. *Nat Rev Genet* 6, 507–512 (2005).
2. McVey, M. & Lee, S. E. MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. *Trends in Genetics* 24, 529–538 (2008).
3. Stoddard, B. L. Homing endonuclease structure and function. *Quart. Rev. Biophys*. 38, 49 (2006).
4. Montoliu, L. What is CRISPR-Cas9? *The CRISPR page at CNB* http://wwwuser.cnb.csic.es/~montoliu/CRISPR/ (2019).
5. Maeder, M. L. *et al.* Rapid "Open-Source" Engineering of Customized Zinc-Finger Nucleases for Highly Efficient Gene Modification. *Molecular Cell* 31, 294–301 (2008).
6. Boch, J. TALEs of genome targeting. *Nat Biotechnol* 29, 135–136 (2011).
7. Beumer, K. J. & Carroll, D. Targeted genome engineering techniques in Drosophila. *Methods* 68, 29–37 (2014).
8. Sander, J. D. *et al.* Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nat Biotechnol* 29, 697–698 (2011).
9. Haun, W. *et al.* Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. *Plant Biotechnol J* 12, 934–940 (2014).
10. Jinek, M. *et al.* A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* 337, 816–821 (2012).

11. Mali, P. *et al.* RNA-Guided Human Genome Engineering via Cas9. *Science* 339, 823–826 (2013).

12. Cong, L. *et al.* Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* 339, 819–823 (2013).

13. Mojica, F. J. M., Ferrer, C., Juez, G. & Rodríguez-Valera, F. Long stretches of short tandem repeats are present in the largest replicons of the Archaea Haloferax mediterranei and Haloferax volcanii and could be involved in replicon partitioning. *Molecular Microbiology* 17, 85–93 (1995).

14. Mojica, F. J. M. & Garrett, R. A. Discovery and Seminal Developments in the CRISPR Field. in *CRISPR-Cas Systems: RNA-mediated Adaptive Immunity in Bacteria and Archaea* (eds. Barrangou, R. & van der Oost, J.) 1–31 (Springer, 2013). doi:10.1007/978-3-642-34657-6_1.

15. Mojica, F. J. M., Díez-Villaseñor, C., García-Martínez, J. & Soria, E. Intervening Sequences of Regularly Spaced Prokaryotic Repeats Derive from Foreign Genetic Elements. *J Mol Evol* 60, 174–182 (2005).

16. Barrangou, R. *et al.* CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science* 315, 1709–1712 (2007).

17. Marraffini, L. A. & Sontheimer, E. J. CRISPR Interference Limits Horizontal Gene Transfer in Staphylococci by Targeting DNA. *Science* 322, 1843–1845 (2008).

18. Garneau, J. E. *et al.* The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* 468, 67–71 (2010).

19. Doudna, J. A. & Charpentier, E. The new frontier of genome engineering with CRISPR-Cas9. *Science* 346, (2014).

20. Slaymaker, I. M. *et al.* Rationally engineered Cas9 nucleases with improved specificity. *Science* 351, 84–88 (2016).

21. Doench, J. G. *et al.* Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat Biotechnol* 34, 184–191 (2016).

22. Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A. & Liu, D. R. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 533, 420–424 (2016).

23. Gaudelli, N. M. *et al.* Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. *Nature* 551, 464–471 (2017).

24. Anzalone, A. V. *et al.* Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 576, 149–157 (2019).

25. Adli, M. The CRISPR tool kit for genome editing and beyond. *Nature Communications* 9, 1911 (2018).

26. Young, J. K. *et al.* The repurposing of type I-E CRISPR-Cascade for gene activation in plants. *Communications Biology* 2, 1–7 (2019).

27. Klompe, S. E., Vo, P. L. H., Halpin-Healy, T. S. & Sternberg, S. H. Transposon-encoded CRISPR–Cas systems direct RNA-guided DNA integration. *Nature* 571, 219–225 (2019).

28. Koonin, E. V., Makarova, K. S. & Zhang, F. Diversity, classification and evolution of CRISPR-Cas systems. *Curr Opin Microbiol* 37, 67–78 (2017).

29. Shalem, O. *et al.* Genome-Scale CRISPR-Cas9 Knockout Screening in Human Cells. *Science* **343**, 84–87 (2014).

30. Sanjana, N. E. Genome-scale CRISPR pooled screens. *Analytical Biochemistry* 532, 95–99 (2017).

31. Canver, M. C. *et al.* Variant-aware saturating mutagenesis using multiple Cas9 nucleases identifies regulatory elements at trait-associated loci. *Nat. Genet.* 49, 625–634 (2017).

32. Zhang, R. *et al.* A CRISPR screen defines a signal peptide processing pathway required by flaviviruses. *Nature* 535, 164–168 (2016).

33. Peters, J. M. *et al.* A Comprehensive, CRISPR-based Functional Analysis of Essential Genes in Bacteria. *Cell* 165, 1493–1506 (2016).

34. Garreta, E., Marco, A., Izpisua Belmonte, J. C. & Montserrat, N. Genome editing in human pluripotent stem cells: a systematic approach unrevealing pancreas development and disease. *Stem Cell Investigation* 3, (2016).

35. Volpato, V. & Webber, C. Addressing variability in iPSC-derived models of human disease: guidelines to promote reproducibility. *Disease Models & Mechanisms* 13, (2020).

36. Liu, G.-H. *et al.* Progressive degeneration of human neural stem cells caused by pathogenic LRRK2. *Nature* 491, 603–607 (2012).

37. Ben Jehuda, R., Shemer, Y. & Binah, O. Genome Editing in Induced Pluripotent Stem Cells using CRISPR/Cas9. *Stem Cell Rev and Rep* 14, 323–336 (2018).

38. Hockemeyer, D. & Jaenisch, R. Induced Pluripotent Stem Cells Meet Genome Editing. *Cell Stem Cell* 18, 573–586 (2016).

39. Liu, C., Oikonomopoulos, A., Sayed, N. & Wu, J. C. Modeling human diseases with induced pluripotent stem cells: from 2D to 3D and beyond. *Development* 145, (2018).

40. Artegiani, B. *et al.* Fast and efficient generation of knock-in human organoids using homology-independent CRISPR–Cas9 precision genome editing. *Nature Cell Biology* 22, 321–331 (2020).

41. Montserrat, N., Garreta, E. & Belmonte, J. C. I. Regenerative strategies for kidney engineering. *The FEBS Journal* 283, 3303–3324 (2016).

42. Yusa, K. *et al.* Targeted gene correction of α1-antitrypsin deficiency in induced pluripotent stem cells. *Nature* 478, 391–394 (2011).

43. Wu, S. M. & Hochedlinger, K. Harnessing the potential of induced pluripotent stem cells for regenerative medicine. *Nat. Cell Biol.* **13**, 497–505 (2011).

44. Redfern, W. S. *et al.* Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development. *Cardiovasc Res* 58, 32–45 (2003).

45. Braam, S. R. *et al.* Prediction of drug-induced cardiotoxicity using human embryonic stem cell-derived cardiomyocytes. *Stem Cell Res* **4**, 107–116 (2010).

46. Gabdoulline, R. *et al.* Differences in the Early Development of Human and Mouse Embryonic Stem Cells. *PLOS ONE* **10**, e0140803 (2015).

47. Plaza Reyes, A. & Lanner, F. Towards a CRISPR view of early human development: applications, limitations and ethical concerns of genome editing in human embryos. *Development* 144, 3–7 (2017).

48. Abdelaal, A. S. & Yazdani, S. S. Development and use of CRISPR in industrial applications. in *Genome Engineering via CRISPR-Cas9 System* 177–197 (Elsevier, 2020). doi:10.1016/B978-0-12-818140-9.00016-7.

49. Singh, V. & Dhar, Pawan K. *Genome Engineering via CRISPR-Cas9 System*. (Academic Press, Elsevier, 2020).

50. Yao, R. *et al.* CRISPR-Cas9/Cas12a biotechnology and application in bacteria. *Synthetic and Systems Biotechnology* 3, 135–149 (2018).

51. Bikard, D. *et al.* Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. *Nat. Biotechnol.* 32, 1146–1150 (2014).

52. de la Fuente-Núñez, C. & Lu, T. K. CRISPR-Cas9 technology: applications in genome engineering, development of sequence-specific antimicrobials, and future prospects. *Int Bio (Cam)* 9, 109–122 (2017).

53. Gophna, U., Allers, T. & Marchfelder, A. Finally, Archaea Get Their CRISPR-Cas Toolbox. *Trends in Microbiology* 25, 430–432 (2017).

54. Dhamad, A. E. & Lessner, D. J. A CRISPRi-dCas9 system for archaea and its use to examine gene function during nitrogen fixation by Methanosarcina acetivorans. *bioRxiv* 2020.06.15.153874 (2020) doi:10.1101/2020.06.15.153874.

55. Zhang, G.-C. *et al.* Construction of a Quadruple Auxotrophic Mutant of an Industrial Polyploid Saccharomyces cerevisiae Strain by Using RNA-Guided Cas9 Nuclease. *Appl. Environ. Microbiol.* 80, 7694–7701 (2014).

56. Dinamarca, J., Levitan, O., Kumaraswamy, G. K., Lun, D. S. & Falkowski, P. G. Overexpression of a diacylglycerol acyltransferase gene in Phaeodactylum tricornutum directs carbon towards lipid biosynthesis. *Journal of Phycology* 53, 405–414 (2017).

57. Meyer, R. S., DuVal, A. E. & Jensen, H. R. Patterns and processes in crop domestication: an historical review and quantitative analysis of 203 global food crops. *New Phytol.* 196, 29–48 (2012).

58. Doebley, J. The genetics of maize evolution. *Annu. Rev. Genet.* **38**, 37–59 (2004).

59. Committee on Genetically Engineered Crops: Past Experience and Future Prospects, Board on Agriculture and Natural Resources, Division on Earth and Life Studies & National Academies of Sciences, Engineering, and Medicine. *Genetically Engineered Crops: Experiences and Prospects*. 23395 (National Academies Press, 2016). doi:10.17226/23395.

60. Roychowdhury, R. & Tah, J. Mutagenesis—A Potential Approach for Crop Improvement. in *Crop Improvement: New Approaches and Modern Techniques* (eds. Hakeem, K. R., Ahmad, P. & Ozturk, M.) 149–187 (Springer US, 2013). doi:10.1007/978-1-4614-7028-1_4.

61. FAO/IAEA. Mutant Variety Database. https://mvd.iaea.org/.

62. Abbas, M. S. T. Genetically engineered (modified) crops (Bacillus thuringiensis crops) and the world controversy on their safety. *Egyptian Journal of Biological Pest Control* 28, 52 (2018).

63. EFSA Panel on Genetically modified organisms (GMO). Scientific opinion addressing the safety assessment of plants developed using Zinc

Finger Nuclease 3 and other Site-Directed Nucleases with similar function. *EFSA Journal* 10, 2943 (2012).

64. Breitler, J. C. *et al.* CRISPR/Cas9-mediated efficient targeted mutagenesis has the potential to accelerate the domestication of Coffea canephora. *Plant Cell, Tissue and Organ Culture* 134, 383–394 (2018).

65. Modrzejewski, D. *et al.* What is the available evidence for the range of applications of genome-editing as a new tool for plant trait modification and the potential occurrence of associated off-target effects: a systematic map. *Environ Evid* 8, 27 (2019).

66. Zhang, B. *et al.* A threshold level of oxalate oxidase transgene expression reduces Cryphonectria parasitica-induced necrosis in a transgenic American chestnut (Castanea dentata) leaf bioassay. *Transgenic Res* 22, 973–982 (2013).

67. Kumar, R., Kaur, A., Pandey, A., Mamrutha, H. M. & Singh, G. P. CRISPR-based genome editing in wheat: a comprehensive review and future prospects. *Mol Biol Rep* 46, 3557–3569 (2019).

68. Liu, C.-J., Cai, Y., Zhang, X., Gou, M. & Yang, H. Tailoring lignin biosynthesis for efficient and sustainable biofuel production. *Plant Biotechnology Journal* 12, 1154–1162 (2014).

69. Wang, T., Zhang, H. & Zhu, H. CRISPR technology is revolutionizing the improvement of tomato and other fruit crops. *Horticulture Research* 6, 1–13 (2019).

70. Stein, A. J., Sachdev, H. P. S. & Qaim, M. Potential impact and cost-effectiveness of Golden Rice. *Nature Biotechnology* 24, 1200–1201 (2006).

71. Waheed, M. T. *et al.* Need of cost-effective vaccines in developing countries: What plant biotechnology can offer? *Springerplus* **5**, (2016).

72. Ethics Council of the Max Planck Society *et al.* Discussion paper focusing on the scientific relevance of genome editing and on the ethical, legal and societal issues potentially involved. (2019).

73. Globus, R. & Qimron, U. A technological and regulatory outlook on CRISPR crop editing. *J Cell Biochem* 119, 1291–1298 (2018).

74. Callaway, E. CRISPR plants now subject to tough GM laws in European Union. *Nature* 560, 16–16 (2018).

75. Grohmann, L. *et al.* Detection and Identification of Genome Editing in Plants: Challenges and Opportunities. *Front. Plant Sci.* 10, (2019).

76. Zhang, D. *et al.* Genome editing with the CRISPR-Cas system: an art, ethics and global regulatory perspective. *Plant Biotechnology Journal* 18, 1651–1669 (2020).

77. Kotwica-Rolinska, J. *et al.* CRISPR/Cas9 Genome Editing Introduction and Optimization in the Non-model Insect Pyrrhocoris apterus. *Front. Physiol.* 10, (2019).

78. Zuckermann, M. *et al.* Somatic CRISPR/Cas9-mediated tumour suppressor disruption enables versatile brain tumour modelling. *Nat Commun* 6, (2015).

79. Heckl, D. *et al.* Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing. *Nat Biotechnol* 32, 941–946 (2014).

80. Platt, R. J. *et al.* CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling. *Cell* 159, 440–455 (2014).

81. Whitelaw, C. B. A., Sheets, T. P., Lillico, S. G. & Telugu, B. P. Engineering large animal models of human disease. *The Journal of Pathology* 238, 247–256 (2016).

82. Umeyama, K. *et al.* Generation of heterozygous fibrillin-1 mutant cloned pigs from genome-edited foetal fibroblasts. *Sci Rep* 6, (2016).

83. European Parliament. *Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance*. *276* vol. OJ L (2010).

84. Dickinson, D. J. & Goldstein, B. CRISPR-Based Methods for Caenorhabditis elegans Genome Engineering. *Genetics* 202, 885–901 (2016).

85. Liu, J. *et al.* CRISPR/Cas9 in zebrafish: an efficient combination for human genetic diseases modeling. *Hum Genet* 136, 1–12 (2017).

86. Khan, F. R. & Alhewairini, S. S. Zebrafish (Danio rerio) as a Model Organism. *Current Trends in Cancer Management* (2018) doi:10.5772/intechopen.81517.

87. Alexandratos, N. & Bruinsma, J. World agriculture towards 2030/2050: the 2012 revision. 160 (2012).

88. Burkard, C. *et al.* Pigs Lacking the Scavenger Receptor Cysteine-Rich Domain 5 of CD163 Are Resistant to Porcine Reproductive and Respiratory Syndrome Virus 1 Infection. *Journal of Virology* 92, (2018).

89. Sánchez-Torres, C. *et al.* Expression of porcine CD163 on monocytes/macrophages correlates with permissiveness to African swine fever infection. *Arch. Virol.* 148, 2307–2323 (2003).

90. Gao, Y. *et al.* Single Cas9 nickase induced generation of NRAMP1 knockin cattle with reduced off-target effects. *Genome Biology* 18, 13 (2017).

91. Bharati, J. *et al.* Genome editing in animals: an overview. in *Genomics and Biotechnological Advances in Veterinary, Poultry, and Fisheries* 75–104 (Elsevier, 2020). doi:10.1016/B978-0-12-816352-8.00003-5.

92. Grossman, M. R. Genetically Engineered Animals in the United States: The AquAdvantage Salmon. *Eur. Food & Feed L. Rev.* 11, 190–200 (2016).

93. Gutiérrez-Adán, A., Behboodi, E., Murray, J. D. & Anderson, G. B. Early transcription of the SRY gene by bovine preimplantation embryos. *Mol. Reprod. Dev.* 48, 246–250 (1997).

94. Young, A. E. *et al.* Genomic and phenotypic analyses of six offspring of a genome-edited hornless bull. *Nature Biotechnology* 38, 225–232 (2020).

95. Shafran, D., Kodish, E. & Tzakis, A. Organ shortage: the greatest challenge facing transplant medicine. *World J Surg* **38**, 1650–1657 (2014).

96. Yang, L. *et al.* Genome-wide inactivation of porcine endogenous retroviruses (PERVs). *Science* 350, 1101–1104 (2015).

97. Niu, D. *et al.* Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Science* 357, 1303–1307 (2017).

98. Lu, T., Yang, B., Wang, R. & Qin, C. Xenotransplantation: Current Status in Preclinical Research. *Front. Immunol.* 10, (2020).

99. Piña-Aguilar, R. E. *et al.* Revival of extinct species using nuclear transfer: hope for the mammoth, true for the Pyrenean ibex, but is it time for 'conservation cloning'? *Cloning Stem Cells* 11, 341–346 (2009).

100. Shultz, D. Should we bring extinct species back from the dead? *Science* (2016) doi:10.1126/science.aah7343.

101. Noble, C., Adlam, B., Church, G. M., Esvelt, K. M. & Nowak, M. A. Current CRISPR gene drive systems are likely to be highly invasive in wild populations. *eLife* **7**,.

102. Scudellari, M. Self-destructing mosquitoes and sterilized rodents: the promise of gene drives. *Nature* 571, 160–162 (2019).

103. Shahryari, A. *et al.* Development and Clinical Translation of Approved Gene Therapy Products for Genetic Disorders. *Front. Genet.* 10, (2019).

104. Dunbar, C. E. *et al.* Gene therapy comes of age. *Science* 359, (2018).

105. Li, H. *et al.* Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. *Sig Transduct Target Ther* **5**, 1 (2020).

106. Li, C., Mei, H. & Hu, Y. Applications and explorations of CRISPR/Cas9 in CAR T-cell therapy. *Brief Funct Genomics* 19, 175–182 (2020).

107. Mullard, A. Gene-editing pipeline takes off. *Nature Reviews Drug Discovery* 19, 367–372 (2020).

108. Elverum, K. & Whitman, M. Delivering cellular and gene therapies to patients: solutions for realizing the potential of the next generation of medicine. *Gene Therapy* 1–8 (2019) doi:10.1038/s41434-019-0074-7.

109. Ledford, H. CRISPR gene editing in human embryos wreaks chromosomal mayhem. *Nature* 583, 17–18 (2020).

110. Ledford, H. CRISPR babies: when will the world be ready? *Nature* 570, 293–296 (2019).

111. Ledford, H. 'CRISPR babies' are still too risky, says influential panel. *Nature* (2020) doi:10.1038/d41586-020-02538-4.

112. Committee on Human Gene Editing: Scientific, Medical, and Ethical Considerations, National Academy of Sciences, National Academy of Medicine & National Academies of Sciences, Engineering, and Medicine. *Human Genome Editing: Science, Ethics, and Governance*. 24623 (National Academies Press, 2017). doi:10.17226/24623.

113. Cyranoski, D. The CRISPR-baby scandal: what's next for human gene-editing. *Nature* 566, 440–442 (2019).

114. Dennis, C. Deaf by design. *Nature* 431, 894–896 (2004).

115. Sandel, M. J. The Case Against Perfection. *The Atlantic* (2004).

116. Savulescu, J. & Bostrom, N. *Human enhancement*. (Oxford Univ. Press, 2010).

117. Murray, T. H. Reflections on the ethics of genetic enhancement. *Genetics in Medicine* 4, 27–32 (2002).

118. McKinsey Global Institute *et al. The Bio Revolution: Innovations transforming economies, societies, and our lives*. https://www.mckinsey.com/industries/pharmaceuticals-and-medical-products/our-insights/the-bio-revolution-innovations-transforming-economies-societies-and-our-lives (2020).

119. Cohen, J. The latest round in the CRISPR patent battle has an apparent victor, but the fight continues. *Science* (2020) doi:10.1126/science.abe7573.

120. Egelie, K. J., Graff, G. D., Strand, S. P. & Johansen, B. The emerging patent landscape of CRISPR–Cas gene editing technology. *Nature Biotechnology* 34, 1025–1031 (2016).

121. Gyngell, C., Bowman-Smart, H. & Savulescu, J. Moral reasons to edit the human genome: picking up from the Nuffield report. *Journal of Medical Ethics* 45, 514–523 (2019).

122. Friedmann, T. Genetic therapies, human genetic enhancement, and … eugenics? *Gene Therapy* 26, 351–353 (2019).

123. Santaló, J. & Casado, M. *Document sobre bioètica i edició genòmica en humans*. (Publicacions i Edicions de la Universitat de Barcelona, 2016).

10. SUMMARY OF THE CYCLE OF CONFERENCE PRESENTATIONS

Why has gene editing revolutionized biology?

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16th September 2019

The genetic information of a living organism can be modified to change its characteristics. Using various genetic editing techniques, we can select a region of the genome and alter it. In the future, these methods could be used to cure diseases, but at present they remain unsafe for use in humans.

DNA is found in the nucleus of the double-stranded eukaryotic cell. The set of DNA in an organism's cell is known as a genome and a specific region of that genome, which contains certain information, is known as a gene. To be able to edit a gene, we first need to locate it within the genome. In the case of CRISPR-Cas9, currently the most widely used genetic editing technique, an RNA molecule guides and searches for the target gene. Once located, the Cas protein cuts the double strand of DNA and the modifications we are interested in can be introduced by the cellular machinery itself as part of the process of completing the sequence and closing the double helix again.

Genetic editing, in general, and CRISPR, in particular, are very useful for laboratory research, as they allow animal models to be generated very easily. Thus, to investigate a human disease caused by an alteration in a given gene, the same genetic alteration can be copied in an animal and used to test treatments.

Animal avatars of a particular person can also be created to specifically study what the best treatment for that individual is. In the field of biotechnology, genetic engineering can also be very useful, as it can increase the yield and production of animals and plants or adapt animal organs so that they can be transplanted into humans.

Before CRISPR-Cas9, other gene-editing techniques, such as zinc fingers or meganucleases, were used, but CRISPR is the one that facilitates gene editing most accurately, economically, and simply. All of these techniques were discovered by studying microorganisms that use them as a defense strategy against viral infections. A wide variety of bacteria are currently being investigated with the aim of finding techniques that are even more efficient than CRISPR, as it is not without its limitations. The first is that it may fail to locate the target gene and accidentally edit a gene that is not the desired target, and the second is that the editions are introduced in the genomes of only certain cells and not in others, causing the body to present a phenomenon known as mosaicism.

For all these reasons, it is still too early to use CRISPR in human therapy safely. However, last year, in China, the human embryos of twins were modified so that they would not become infected with the AIDS virus, of which the father was the carrier. This treatment was irresponsible, since it was not the best alternative available (given the risk of mosaicism, the possibility of altering genes other than the target, etc.). Additionally, several *ex-vivo* clinical studies are being conducted, in which patients' cells are extracted, modified, and reintroduced. This option should be safer, but no promising results have yet to be reported.

Europe's ARRIGE works to regulate the use and marketing of genetic editing tools, and to raise any ethical issues arising from their use. In Spain, the law only allows these techniques to be used to conduct research aimed at preventing or curing diseases, but not to improve capabilities; yet, each country has its own legislative guidelines. Moreover, the fact that the components can be purchased online can lead to their being misused.

Bibliography:

Lluís Montoliu, "*Editando genes: recorta, pega y colorea*", 2019, Next Door Publishers.

Genome editing of somatic cells and pluripotent stem cells: therapeutic applications

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16th December 2019

Pluripotent stem cells, either derived from human embryos or obtained artificially by somatic reprogramming, can differentiate into any cell type in our body. This inherent property, known as pluripotency, has been exploited for two decades to generate fundamental knowledge in the field of organ development and regeneration. In recent years, several studies have shown that the instructions that embryonic stem cells receive during development can be emulated. In this way, the biochemical and, in some cases, physical signals that dictate the specification of the tissues and their correct development have been described.

This knowledge combined with genetic engineering has made it possible to introduce patient-specific genetic mutation in cells derived in the culture plate, generating platforms for applications in precision medicine. At the same time, when patients' reprogrammed cells undergo genetic editing, we can correct mutations and establish "patient-specific" platforms for applications in personalized medicine.

Given that researchers can now also generate micro-organs from pluripotent stem cells, the application of genetic editing technologies in conjunction with this technology is currently opening the door to the study of more complex mechanisms at the multicellular level. In this sense, the application of bioengineering technologies, such as 3D bioprinting or microfluidic systems, is leading to the generation of micro-organs, or organoids that have complex characteristics and functions. In this talk, we discuss how the above findings are bringing the application of this fundamental knowledge much closer to clinical practice.

Gene editing in somatic cells: therapeutic applications

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16th December 2019

Although from a technical point of view genome editing in cells of the somatic or germinal lines may not present great differences, the consequences and implications of both manipulations differ radically. A change in the genome of any somatic cell spreads only to the offspring of that cell, giving rise, when carried out in a person (as in the case of gene therapy discussed below), to an organ or tissue mosaic, in the sense that it contains both modified and unmodified cells. Yet, the persistence of this modification is limited, at most, to the duration of the individual's life. In contrast, manipulation of the genome of a germ cell results in all the cells of the individual derived from it containing the manipulation, including its germ cells (or at least half of them). In this way, the manipulation is passed on to the individual's offspring. In this presentation, we focus mainly on somatic cell gene editing and its therapeutic implications, leaving those associated with germ cell editing for another occasion.

Technically, introducing "extra" genes into a cell's genome is relatively straightforward. These "extra" genes are known as transgenes and the organisms containing them are, therefore, transgenic. The technology that allows the generation of transgenic organisms was developed in the laboratory in mice during the early 1980s (Brinster *et al.*, 1981; Costantini and Lacy, 1981; Gordon and Ruddle, 1981). Using this technology, injecting transgenes (in the form of naked DNA) into very early mouse embryos, when they still have a single cell, causes them to be randomly integrated into the genome of the animal's cells. In most cases, the animal is a mosaic of cells that contain the transgene and others that do not. However, if the transgene has integrated into cells of the animal's germ line (those that give rise to sperm and eggs), its offspring will be completely transgenic.

This technology has since left the laboratory to become a medical application in the form of gene therapy. In this case, we are not trying to generate a completely transgenic individual, rather only some of its cells, to which a transgene is added using a special type of virus. After a long and eventful phase of development, the efficacy and safety of gene therapy for treating diseases through the random insertion of transgenes into the somatic cell genome has been successfully demonstrated (Aiuti *et al.*, 2013; Biffi *et al.*, 2013).

If the introduction of random transgenes into a cell's genome seems relatively straightforward, their removal presents extraordinary technical difficulties, because it is necessary to act on a specific site of the 3 billion bases making up

the genome, that is, in a targeted fashion. The technology needed to do this was developed thanks to the conjunction of two truly revolutionary developments: On the one hand, the obtention of embryonic mouse stem cells, which can be maintained in culture in the laboratory and which are capable of generating a whole mouse (Evans and Kaufman, 1981; Martin, 1981); and, on the other, the possibility of carrying out directed modifications in the genome of a cell, through a process known as "homologous recombination" (Smithies *et al.*, 1985; Thomas *et al.*, 1986). If we combine both developments, we are able to modify the genome of mouse embryonic stem cells in a targeted manner and, consequently, generate mice from these cells, the genome of which is modified. For these discoveries, which made possible the generation of animals with directed modifications in their genome (commonly called *knockout* mice), Mario Capecchi, Martin Evans and Oliver Smithies received the Nobel Prize in Physiology or Medicine in 2007. This technology has since been used to identify the function (by loss-of-function experiments) of thousands of genes. Over time, it has been perfected not only in order to eliminate genes, but also to introduce any type of modification of interest in the genome.

The great limitation of this technology is that its application was, until very recently, virtually exclusive to laboratory mice. Essentially, and in very simple terms, this was the case because the process of homologous recombination is extraordinarily infrequent. The development of methods to increase the likelihood of homologous recombination occurring has gone a long way to removing these limitations. To achieve this, a break is generated in the DNA strands at the very site of the genome we wish to modify and, taking advantage of the cell endogenous machinery that normally repairs these breaks, we trick it into repairing the cut in the way that we want. Over the past 20 years, several tools have been developed to cut DNA in a targeted manner, including zinc finger nucleases and TALENs (a story of their development can be found in Baker, 2012). However, the advent of CRISPR-Cas9 (Doudna and Fuster, 2014) has provided an effective, much more versatile, and simple method of causing DNA breakage at virtually any point in the genome. It is so simple, in fact, that it is now possible to make changes aimed at "editing" the genome, not just in mouse embryonic stem cells, but in virtually any cell of any species.

The enhanced ability to edit the genome of a somatic cell greatly increases the therapeutic applications of gene therapy. Thus, not only can diseases caused by the loss of function of a gene be treated, as is currently the case with transgenic gain-of-function, but a large number of diseases attributable to other genetic causes can also be addressed. It is not expected that the implementation of these strategies to treat patients (in all likelihood using CRISPR-Cas9-based tools) will encounter any greater obstacles than those arising from their technical development and application in humans. Given the speed at which advances in this field are taking place, it is expected that in the next 5 years this type

of treatment based on the genetic editing of somatic cells will be clinically available.

Given the basic developments in pre-clinical and ongoing clinical trials, the identity of the diseases set to benefit from gene editing therapies in somatic cells can be reasonably predicted. At present, CRISPR-Cas9-based tools achieve low or moderate genome editing efficiencies, and as long as this continues, the most rapidly transportable therapeutic strategies in clinical practice will be loss-of-function. They aim to cut the DNA of the cell at a specific site in a specific gene, so that this break is repaired at random and by introducing random changes that are likely to destroy the function of the gene. Such strategies have been designed for diseases such as sickle cell anemia and beta thalassemia (Wu *et al.*, 2019), and a phase I/II clinical trial is currently being conducted for sickle cell anemia, led by Vertex Pharmaceuticals and CRISPR Therapeutics, whose final results are expected in May 2022. This type of random repair can also be applied in situations where cells that have the gene edited correctly achieve a selective advantage over uncorrected genes, as in the case of Fanconi's anemia (Roman-Rodriguez *et al.*, 2019). Finally, this type of strategy can be expected to be applied clinically in the short term in monogenic diseases in which the affected cells are particularly accessible to existing gene therapy tools, such as Leber's congenital amaurosis, in which a first patient enrolled for an ongoing clinical trial has already been treated (Ledford, 2020).

As CRISPR-Cas9-based tools improve and high efficiency in homologous recombination-directed gene editing is achieved, this strategy is expected to replace randomized repair for the aforementioned diseases and its applications are expected to expand to a much larger number of similar conditions. However, its use is unlikely to exceed the scope of action of current gene therapy for monogenic minority diseases. The reason for this relative pessimism is that the introduction of CRISPR-Cas9-based gene editing tools, although an outstanding innovation, in the context of its application to somatic cells remains conditioned by the main limitation of conventional gene therapy, that is, how to deliver these tools specifically to the largest number of disease-relevant somatic cells. Until this limitation is resolved, the field of application will be restricted to somatic cells in which gene editing can take place *ex vivo*, such as blood cells, or those that are especially accessible for the introduction of nucleic acids/protein complexes, such as retinal cells.

In short, the ability to edit the cell genome effectively and selectively has revolutionized our ability to interrogate the genetic bases of many cell biology and developmental processes, as well as to generate genetically modified individuals thanks to the editing of germline cells. However, the therapeutic applications of targeted somatic-cell gene editing are not so notable and are largely restricted by the current limitations of conventional gene therapy.

Bibliography:

Aiuti, A., Biasco, L., Scaramuzza, S., Ferrua, F., Cicalese, M.P., Baricordi, C., Dionisio, F., Calabria, A., Giannelli, S., Castiello, M.C., *et al.* (2013). Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science* 341, 1233151.

Baker, M. (2012). Gene-editing nucleases. *Nat Methods* 9, 23-26.

Biffi, A., Montini, E., Lorioli, L., Cesani, M., Fumagalli, F., Plati, T., Baldoli, C., Martino, S., Calabria, A., Canale, S., *et al.* (2013). Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science* 341, 1233158.

Brinster, R.L., Chen, H.Y., Trumbauer, M., Senear, A.W., Warren, R., and Palmiter, R.D. (1981). Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. Cell 27, 223-231.

Costantini, F., and Lacy, E. (1981). Introduction of a rabbit beta-globin gene into the mouse germ line. *Nature* 294, 92-94.

Doudna, J.A., and Charpentier, E. (2014). Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* 346, 1258096.

Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154-156.

Gordon, J.W., and Ruddle, F.H. (1981). Integration and stable germ line transmission of genes injected into mouse pronuclei. *Science* 214, 1244-1246.

Ledford, H. (2020). CRISPR treatment inserted directly into the body for first time. *Nature* 579, 185.

Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* 78, 7634-7638.

Roman-Rodriguez, F.J., Ugalde, L., Alvarez, L., Diez, B., Ramirez, M.J., Risueno, C., Corton, M., Bogliolo, M., Bernal, S., March, F., *et al.* (2019). NHEJ-Mediated Repair of CRISPR-Cas9-Induced DNA Breaks Efficiently Corrects Mutations in HSPCs from Patients with Fanconi Anemia. *Cell Stem Cell* 25, 607-621 e607.

Smithies, O., Gregg, R.G., Boggs, S.S., Koralewski, M.A., and Kucherlapati, R.S. (1985). Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature* 317, 230-234.

Thomas, K.R., Folger, K.R., and Capecchi, M.R. (1986). High frequency targeting of genes to specific sites in the mammalian genome. *Cell* 44, 419-428.

Wu, Y., Zeng, J., Roscoe, B.P., Liu, P., Yao, Q., Lazzarotto, C.R., Clement, K., Cole, M.A., Luk, K., Baricordi, C., *et al.* (2019). Highly efficient therapeutic gene editing of human hematopoietic stem cells. *Nat Med* 25, 776-783.

Plant genome editing: its application in plant improvement and its regulation in Europe

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20th January 2020

The ability to specifically modify the genomic sequence of organisms has applications in many different fields. Its use in medicine to try to correct mutations linked to genetic diseases is probably the one that arouses most interest in society; however, applications to the genetic improvement of plants are the ones that are likely to enter our lives and reach our markets first.

In this communication, I explain the interest of gene-editing techniques for plant genetic improvement and compare them with other techniques used such as selective breeding, radiation mutagenesis, and transgenesis. Genetically edited plants are already a reality in laboratories around the world, and the products obtained from these plants are already being marketed in some countries. But the transition from laboratory to market is not easy and the analysis of the possible risks arising from the use of these techniques can involve significant investments of both time and money. This can significantly limit both the type of plant or character to be improved and the type of company that is able to do the work. Therefore, in this communication, I also review the regulations adopted in different countries that apply to the commercialization of plant-derived products obtained by new improvement techniques, paying special attention to the situation in Europe.

The impact of genome editing in agriculture

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20th January 2020

Climate change and population growth pose threats to environmental sustainability and food security that must be addressed from all possible angles. One of them is the genetic improvement of plants. The genetic adaptation of our crops to new environmental demands is within our reach to respond to these threats. Plant genetic improvement can help, among other things, reduce the use of potentially harmful substances such as pesticides, increase crop yields, reduce water use, facilitate the replacement of plastics for biodegradable polymers and produce value-added molecules in a sustainable fashion and with a lower carbon footprint.

In recent times, the boundaries between traditional breeding based on sexual intercourse and random mutagenesis, on the one hand, and transgenic breeding based on molecular biology techniques, on the other, have largely disappeared with the advent of the so-called new plant breeding techniques (NPBTs) based on genetic improvement, ranging from cis-genesis (transferring genes between closely related species) to synthetic biology. The most striking of these NPBTs is undoubtedly genetic editing based on site-specific nucleases (SSN) and, especially, the CRISPR-Cas technology, which has been successfully applied as a mutagenic agent guided by RNA in many different culture species.

The power of CRISPR-Cas as a tool for improvement speaks for itself in the dozens of examples of goals successfully achieved in the six years that the technique has been applied to plants. In fact, as well as improving traditional crops, CRISPR-Cas9 technology is also being used in the rapid design of new crops for innovative sustainability-related applications, such as the use of plants for manufacturing drugs and the adaptation of field crops to the needs of urban agriculture. In addition, the intense research effort that followed the initial discovery of CRISPR-Cas and its promising biotechnological applications has led, as in a self-fulfilling prophecy, to the development of new CRISPR-Cas-based tools with expanded uses and applications. This has been possible thanks to the prodigious ability of CRISPR-Cas ribonucleoproteins to accept the adhesion of new protein modules that provide additional functions.

In this talk, we review some of the most recent and most innovative examples of genetic improvement based on CRISPR, some of which would have been difficult if not impossible to achieve using traditional breeding techniques. In addition, we discuss some of the expanded functions of CRISPR-Cas proteins and their applications to plant biotechnology, from multiplexed mutagenesis to programmable transcriptional regulation.

Precise engineering of the mammalian genome

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20th April 2020

Over the last decade, our capacity to engineer genomes has increased significantly impacting biomedical research and medicine. Despite important progress, mammalian genome engineering still faces important challenges such as limited multiplexability and the difficulty to generate large edits efficiently. In this briefing, I present our work using CRISPR-Cas9 technologies to create pigs free of porcine endogenous retroviruses (PERVs) for xenotransplantation after tens of simultaneous edits.

In the US alone, twenty people die each day waiting for an organ (United Network for Organ Sharing, UNOS, 2020). This lack of organs for xenotransplantation is one of the biggest unmet medical needs. Two problems hinder xenotransplantation: the presence of PERVs in the genomes of pigs and pig-to-human compatibility. Modern gene-editing techniques are being used to tackle both problems with the objective of producing an unlimited supply of organs in genetically modified pigs. The progress to date has been astonishing. Pigs free of endogenous retroviruses have been produced (I) and genetically modified pigs' organs survive in non-human primate models for years (II).

I. PERVs are a major concern for xenotransplantation applications as they can be transferred from pigs to humans (Güell *et al.*, 2017). Since they are integrated into the genome, they are present in all tissues and organs and are transmitted vertically from parents to offspring. Genome editing enables us to remove or inactivate PERVs, thus making genetically modified pigs' organs suitable for xenotransplantation. In 2015, we reported the use of CRISPR-Cas9 to eliminate 62 copies of PERVs in the pig genome (Yang *et al.*, 2015) and demonstrated more than a 1000-fold reduction in PERV transmission to human cells. This finding shows that CRISPR-Cas09 multiplexability can be as high as tens of edits and that PERVs can be inactivated for clinical application of porcine-to-human xenotransplants. In 2017, we went on to inactivate all PERVs (Niu *et al.*, 2017) and to produce pigs which appeared healthy and fertile with functioning organs. Recently, the team has begun to transplant organs from the highly edited pigs into non-human primates to gauge their safety and longevity.
II. Immune and physiological engineering to increase pig-to-human compatibility is also performed by genetic engineering. Important human genes are added to the pig genome and genes that pigs and humans do not have are removed. We have already seen pigs that can produce hearts (Längin *et al.*, 2018), kidneys (Iwase *et al.*, 2017) and pancreatic islets (Aristizabal *et al.*, 2017) that last for years in non-human primates.

Editing pig genomes for xenotransplantation would probably address the biggest unmet medical need today, which is providing an unlimited supply of organs for patients on a transplant waiting list.

Bibliography:

Aristizabal, A. M. *et al.* (2017) 'Clinical Xenotransplantation, a Closer Reality: Literature Review', *Cirugía Española* (English Edition), 95(2), pp. 62–72. doi: 10.1016/j.cireng.2017.03.007.

Güell, M. *et al.* (2017) 'PERV inactivation is necessary to guarantee absence of pig-to-patient PERVs transmission in xenotransplantation', *Xenotransplantation*, 24(6), p. e12366. doi: 10.1111/xen.12366.

Iwase, H. *et al.* (2017) 'Immunological and physiological observations in baboons with life-supporting genetically engineered pig kidney grafts', *Xenotransplantation*, 24(2). doi: 10.1111/xen.12293.

Längin, M. *et al.* (2018) 'Consistent success in life-supporting porcine cardiac xenotransplantation', *Nature*, 564(7736), pp. 430–433. doi: 10.1038/s41586-018-0765-z.

Niu, D. *et al.* (2017) 'Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9', *Science* (New York, N.Y.), 357(6357), pp. 1303–1307. doi: 10.1126/science.aan4187.

United Network for Organ Sharing, UNOS (2020) 'Transplant trends'. Available at: https://unos.org/data/transplant-trends/ (Accessed: 1 September 2020).

Yang, L. *et al.* (2015) 'Genome-wide inactivation of porcine endogenous retroviruses (PERVs)', *Science*, 350(6264), pp. 1101–1104. doi: 10.1126/science.aad1191.

CRISPR-Cas9 gene editing of mice embryos

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20th April 2020

The use of genetically modified mice as models of human disease and for investigating the function of specific genes and mechanisms that occur *in vivo* is a widely employed tool in research projects. Transgenic mice have been generated since the 1980s. Recently, thanks to the use of CRISPR-Cas9 gene-editing techniques, the creation of transgenic mice can be undertaken more efficiently, which has opened the door to the use of new methodologies for generating transgenic mice. Today, this system is so efficient that it is possible to generate transgenic mice without the need to resort to complicated microinjection techniques. More recently, it has been shown that CRISPR-Cas9 allows the generation of transgenic mice *in vivo*, when the embryos are still in the oviduct before implantation. This opens up the possibility of generating transgenic animals from other species of interest. In this communication, we explain our experience in the generation of transgenic mice using CRISPR-Cas9 technology.

Genome editing in *Caenorhabditis elegans*

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20th April 2020

The nematode *C. elegans* was first adopted in 1974 as a model organism and, since then, the worm has been essential in the advancement of genetics and developmental biology. It was the first animal to have its whole genome sequenced and approximately 42% of its 20,000 genes have human orthologs, many of which are associated with disease. Therefore, its use has also recently expanded into biomedical research and personalized medicine.

Several features make *C. elegans* an attractive organism for gene editing. First, it has a short life cycle of 3 to 5 days, depending on rearing temperature (usually from 15 °C to 25 °C). Second, as they mainly exist as hermaphrodites, genetic crosses are not needed to isolate homozygous offspring from heterozygous parents. And third, its syncytial germline (cells with multiple nucleoli) allows the simultaneous editing of many nuclei in a single microinjection.

Precise changes in the worm's genome can be achieved in two to three weeks with relative ease using the CRISPR-Cas system, ranging from point mutations, deletions, or the insertion of large fragments of DNA, as in the case of sequences encoding for GFP or other fluorescent proteins. The latter is of great interest in the *C. elegans* community due to its transparency, allowing researchers to track the expression levels and subcellular localization of proteins over time, making the use of fluorescent reporters a useful tool for the analysis of gene functions.

C. elegans is amenable to transgenesis, which was commonly carried out via microinjection of exogenous DNA that form extrachromosomal arrays or integrated via biolistic transformation. However, the advent of CRISPR-Cas technology has practically phased these methods out. Gene editing via CRISPR-Cas facilitates gene editing at endogenous loci, allowing the study of gene expression at native levels. Random mutations can be created through the non-homologous end joining (NHEJ) pathway, or specific changes such as gene replacement or the introduction of tags can be achieved through homology-directed repair (HDR). This can be done in a reproducible manner since most of the required materials and reagents are commercially available.

In line with the 3Rs principle for the more ethical use of animals in testing, *C. elegans* is a plausible alternative to vertebrate animal models. Since it is an invertebrate, it does not fall within the scope of the European Directive 2010/63/EU and, thus, ethical concerns do not pose a barrier for using thousands of *C. elegans* individuals for performing genetic and drug screens in preclinical studies.

In summary, the tractable genetic system of *C. elegans* coupled with its short life cycle allows the generation of homozygous mutants within several days, a feature that is unrivaled by other model organisms such as *Drosophila*, zebrafish, or mice. These genetically modified worms can be used as avatars for mimicking human mutations, thereby providing access to personalized diagnosis and treatment in a rapid, scalable, and cost-effective manner.

Natural dynamics of evolving genomes

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4th May 2020

One of the points of reflection in discussing the artificial modification of genomes is their dynamics, what we might call a genome's natural or, rather, spontaneous dynamics. Genomes change over time and across generations and this feature is the basis for the existence of life as we know it: the origins of the diversity of life that exists (including all that has been lost) lie in the production of new variants by processes of mutation. Mutation, as a consubstantial phenomenon of life, occurs actively over the course of cell generations, affecting genomes in different ways, including substitutions, insertions, deletions, translocations, and so on. Here, a clear distinction should always be drawn between somatic mutations (which affect any non-reproductive cell of an organism) and germinal mutations (which affect reproductive cells and, therefore, the whole organism in the next generation). While the former is of importance to the individual, the latter is critical to evolution.

More importantly, their dynamics affects genomes on all time scales: while there have obviously been changes in genomes throughout evolution, it is no less true that changes also occur in each generation. On this short time scale, change can now be observed and measured. Thus, if we consider only nucleotide substitutions (those affecting just one unit of the genetic information that we carry), various studies have calculated the mutation rate that exists in a single generation (of the order of 10^{-8} per nucleotide) and this, given the size of our genome ($3*10^9$ nucleotides), allows us to estimate that each of us carries, on average, about 60 new variants, 30 from each parent. We are, therefore, mutants and each of us carries a considerable amount of genomic novelty that may have different futures: much of this genomic novelty has no effect on the individual and variations over generations are exclusively random and just as likely to be lost; others have detrimental effects and are at the root of genetic disease, with purifying selection reducing its frequency; and lastly, a very small number may be the basis of a new adaptation, and positive selection will increase their frequency.

Our genome comprises much more than only these substitutions, there being a very rich internal dynamic that affects larger parts of the genome. Almost 50% of our genome is made up of repetitive regions. Among them, transposable elements (TEs) are DNA sequences that have the ability to change their position within a genome. The genome can be thought of as an ecosystem inhabited by various TE communities, which seek to propagate and multiply through sophisticated interactions. These interactions encompass familiar processes for ecologists, such as parasitism, cooperation, and competition. Transposition represents a powerful mechanism of genome expansion that, over time, is counteracted by the removal of DNA by suppression. The balance between the two processes is one of the main drivers of the evolution of genome size in eukaryotes.

The rate of transposition in the human germline has been studied for different types of repetitive elements in our genome. Through the comparison of the high quality genomic sequence between successive generations of individuals, it has been possible to observe the appearance of new repetitive regions in new places in the genome. For instance, considering exclusively Alu sequences (a very common type of DNA sequence of only 280 base pairs), there is approximately one new insertion for every 21 births, with many other elements appearing at lower frequencies. Thus, we can begin to determine the rate and dynamics of human retrotransposition, which shows us that it is a common phenomenon: many of us inaugurate in our species some reorganization of the genome.

These reorganizations include DNA fragments of very varied origin, many of them from other distant species such as bacteria or viruses. Bear in mind, for instance, that the amount of DNA of viral origin in the human genome is roughly five times greater than the DNA that includes our human genes. A genome is not a design product, optimized to perform specific functions of a particular species. It is a set of elements that include evolutionary remains from other times and that change across generations. We are all mutants and we are all transgenic. What we are learning by using genome editing technologies are simply the most basic elements of what genomes naturally use in order to introduce a certain dose of change into the permanence that permeates life.

The germ of gene editing

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4th May 2020

Gene-editing techniques use components of yeast, microscopic algae or, above all, prokaryotes (bacteria and archaea) to carry out a process that culminates in the modification of specific regions of DNA in the genome of a living thing.

These editing strategies usually begin with the interruption of the continuity of the sequence in which the insertion, deletion, or substitution of genetic material is intended. In all cases, from the technology based on meganucleases and that which uses Cas proteins (clustered regularly interspaced short palindromic repeats or CRISPR associated) to those based on zinc finger nucleases (ZFNs) and transcription activator-like effector-based nucleases (TALENs), the cut is made by proteins of microbial origin whose mission in its natural host differs from that for which they have been redesigned in the laboratory. Meganucleases probably serve the genetic element that encodes them to ensure its multiplication and propagation, while the activity of nucleases associated with ZFN, TALEN or CRISPR produces the destruction of invasive nucleic acids, thus protecting the prokaryote organism that carries them from invaders such as viruses.

In this context, native CRISPR-Cas systems are an exceptional defense mechanism, present in most archaea and a little less than half of the known bacteria. CRISPR elements were the first components of these systems to be discovered more than three decades ago. At the beginning of this century, the Cas proteins were identified and, shortly afterwards, their function was established, assisted by the information contained in the CRISPR clusters. Much of the spacing sequences located between CRISPR repeats come from viral genomes, acting as records of past infections. RNAs generated from these regions guide the Cas proteins to recognize these infectious agents and neutralize them by cutting their genome.

It is, therefore, an immune system based on the recognition of nucleic acid sequences, with the ability to adapt by incorporating a spacer from the genome of new invaders into a CRISPR cluster. The artificial generation of this memory, through the *in vitro* synthesis of guide RNA that contains the sequence coinciding with that of the genetic region where the Cas proteins are to be sent, has given rise to an extraordinary set of tools known collectively as CRISPR technology, used for various purposes related to the interaction with nucleic acids and their *in vitro* and *in vivo* manipulation. Among the many applications of this technology implemented in life sciences and health, we should highlight the regulation of gene expression, molecular diagnosis, and genetic editing, in any cell type, from bacteria to human cells, with unprecedented accuracy, efficiency and ease.

The enormous diversity of microorganisms in nature provides an inexhaustible source of laboratory tools. Perhaps among them we shall identify some that are as useful as CRISPR technology and as prodigious as the immune system from which it is derived.

Organoids and genome editing in the study of the phenotypic diversity of colorectal cancer

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Cancers are amalgams of phenotypically different tumor cell populations. As a result of genomic instability, cancers acquire hundreds of genetic and epigenetic alterations that impose different phenotypes on tumor cells. This phenomenon explains the ability of cancer to adapt to different environments, colonize other organs and resist therapy. However, over the years, it has become clear that tumor heterogeneity arises not only from the mutational load, but also from its own phenotypic architecture. In this sense, colorectal cancer (CRC) is a paradigm of the role of stem cells in cancer. The colonic epithelium is constantly being renewed thanks to a population of stem cells that reside at the base of each crypt. Millions of cells are generated every day that undergo functional differentiation near the intestinal lumen. Our laboratory has been studying the organization of CRCs in relation to that of the normal colonic mucosa. In 2011 we described that, despite the acquisition of multiple genetic alterations, most CRCs retain a stem cell hierarchy reminiscent of that in healthy mucosa. We showed that CRCs contain a subset of tumor cells similar to stem cells, which continuously regenerate cancer, while the offspring of these stem cells form the bulk of the tumor, but it is short-lived and not very tumorigenic as a result of differentiation. The two types of cell occupy compartments adjacent to the tumor glands and are present in different proportions in each patient. This organization is common in most CRCs (80%) (Merlos-Suárez *et al.*, 2011).

Two recent technological advances have transformed our ability to dissect tumor heterogeneity in CRC. The first are organoids, three-dimensional (3D) structures cultured *in vitro* and derived from self-organized stem cells. The term organoid is used because these 3D cultures resemble the source organ in terms of self-organization, multicellularity, and functionality. In many respects they can be considered as mini organs. Stem cell-derived 3D organoids are an invaluable research tool that has been rapidly used to understand stem cell biology, organogenesis, and various human pathologies, including cancer. In fact, organoids represent the most current and appropriate technology for the study of relevant events in many types of cancer, including CRC. In addition, they represent an *in vitro* platform for the discovery of pre-clinical drugs and their value in predicting therapy responses is currently being investigated. We developed a method that allowed for the first time both the isolation of human colon stem cells from healthy mucosal biopsies and their culture as constantly

expanding organoids that recreate the organization of colony crypts in the petri dishes (Jung *et al.*, 2011). The protocol was later adapted to grow and preserve tumor stem cells from tumors (Merlos Suàrez *et al.*, 2011; Calon *et al.*, 2015).

The second important development has been our ability to edit the genome of these organoids using the CRISPR-Cas9 technique and derivatives (Cortina *et al.*,2017; Morral *et al.*, 2020). The study of stem cell hierarchies and other sources of cell diversity in human cancers had been largely based on tumor cell isolation experiments from dissociated patient samples. These experiments impose several limitations. First, the requirement for antibodies against specific membrane proteins to label certain cell populations limits the repertoire of cellular phenotypes that can be analyzed. Second, the need to dissociate the sample prevents the examination of tumor cell populations in an intact environment, that is, in a growing tumor.

Combining these two new methodologies – patient-derived tumor organoids and genome editing tools – we can study the cell heterogeneity of CRCs without the limitations described above (Cortina *et al.*, 2017; Morral *et al.*, 2020). CRISPR-Cas9-mediated genome editing facilitates the integration into organoids derived from CRC patients (PDO, patient-derived organoids) of gene sequences within marker genes, allowing the study of human tumors using genetic approaches that had only been feasible in animal models. The edited organoids make it possible to perform classical genetic experiments on clonal expansion, lineage tracing and cell ablation in tumors. This advancement is especially suitable for analyzing the phenotypic diversity of cell populations within cancers, as it allows different tumor cells to be labeled and traced through specific marker genes, which are not necessarily expressed on the cell surface.

As a proof of concept, we studied LGR5+ stem cells in organoids from patients with CCR (PDO, patient-derived organoids), the analysis of which had been hampered by the lack of good commercial reagents to recognize this protein. Using CRISPR-mediated gene editing, we designed PDOs that carried a GFP reporter cassette connected to the LGR5 protein. We found that the LGR5+ tumor cell population expresses a gene program similar to that of normal intestinal stem cells. In tumors grown in mice such as xenographs, human LGR5+ tumor cells spread the disease with high efficiency, implying that this cell population consists largely of tumor-initiating cells. In addition, PDOs carrying a lineage trace cassette were generated and the fate of LGR5+ cells in intact tumors was subsequently mapped. We found that LGR5+ cells show a long-term capacity for self-renewal and multi-lineage differentiation. Finally, by generating double knock-in LGR5-GFP/Ki67-RFP PDOs, we described a quiescent stem cell population in human CRCs (Cortina *et al.*, 2017).

Another example of the potential of the approaches described above is the study of cell plasticity by the elimination of specific cell populations (Cortina

et al., 2017; Morral *et al.*, 2020). Characterizing the properties of cancer stem cells, we found that most of the RNA and proteins synthesized in CRCs occur in a limited subset of cells that reside immediately next to the stroma. In contrast, as tumor cells differentiate, they experience irreversible loss of RNA and protein synthesis capacity. Using strategies of cell ablation, lineage tracing, and RNA sequencing of individual cells based on CRISPR-Cas9, we demonstrated that in a subset of CRCs the compartment of biosynthetic tumor cells fits the domain of expression LGR5 while in other tumors it seems to feed tumor growth without the contribution of LGR5+ cells. Tumor cells exhibit plasticity while maintaining biosynthetic capacity. If they lose it, the differentiation is irreversible. The RNA and protein synthesis localization patterns we describe reflect the existence of a simple stem cell hierarchy based on the differential biosynthetic capacity of tumor cells (Morral *et al.*, 2020).

Bibliography:

Merlos-Suarez *et al.*, Cell Stem Cell 8(5):511-524 2011).

P. Jung *et al.*, Nat Med 17: 1225. 2011.

Calon *et al.*, Nat Genetics 47: 320-362. 2015.

Cortina *et al.*, EMBO Mol Medicine 9: 869-879. 2017.

Morral *et al.*, Cell Stem Cell 26: 845-861. 2020

Germline gene editing

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4th May 2020

Germline genome editing (GGE), which can be performed in pluripotent stem cells, spermatogonic cells, and embryos at different stages of development, has ushered in a new era, especially with the advent of the CRISPR-Cas9 technique and its variants.

This methodology allows the genetic modification of pluripotent stem cells that can subsequently be transformed into both male and female gametes through *in vitro* gametogenesis, and has already been achieved in mice. Sperm stem cell editing allows us to obtain modified sperm, and this has also been achieved in animal models (rat).

GGE could be applied in several specific cases, including couples in which both members are homozygous for a recessive genetic disease, in which one of the members is homozygous for a dominant genetic disease and in cases in which one of the members carries a chromosomal structural alteration (such as translocation 21/21, theoretical indication). In all other cases of couples with genetic diseases, the technique of choice is preimplantation genetic diagnosis so as to select disease-free embryos rather than GGE.

To date, several studies have been published on GGE in human embryos. They began with tripronucleate embryos (zygotes from eggs fertilized by two sperm) and later moved on to embryos from properly fertilized zygotes, donated to research or created specifically for experiments. Legislation can vary greatly between countries and China, the US, and the UK lead the field. These publications demonstrate the possibility of carrying out defective gene repair, insertion, or disruption of genes, where the purpose may be curative, to correct alterations, or to determine the role of certain genes in embryonic development.

The main technical limitations of GGE in embryos are the appearance of off-target phenomena (that is, modifications to sites other than the one intended for modification) and embryonic mosaicism, in which different cells of the embryo are modified differently. Additionally, on-target phenomena have recently been described in which genetic modification does not take place in the precise target of the chosen locus. A complete analysis of the genome of the embryo is needed to assess these phenomena. New studies have recently been published demonstrating the need for GGE optimization in embryos to address loss of heterozygosity, segmental or total chromosome loss, and lack of repair in a high percentage of edited embryos.

Although there is a global consensus not to use GGE for reproductive purposes on the grounds that current technical limitations cannot guarantee the safety of the technique, a report in November 2018 announced the birth of two newborns whose embryos had been genetically modified by a Chinese researcher who chose to ignore all the warnings of the world's scientific and bioethical societies. The embryos were genetically modified to make them inaccessible to HIV infection.

Embryos donated by couples undergoing *in vitro* fertilization and who no longer want them for reproduction and accept they be used for research purposes are an invaluable source for promoting research in the field of GGE. However, the efficiency of this technique and, especially, its safety must be evaluated before considering its possible clinical application.

The thin gray line: Gene editing for human genetic enhancement

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4th May 2020

A large part of human culture probably emerged from a desire to be desirable and to appear at our best in front of our peers. Decorative seashells, gold cuffs and rings, precious stone necklaces, perfume and ointment vessels and ornate textiles are found as funerary goods in all cultures. Women and men alike have used make-up to enhance their beauty and facial expressions and as a sign of their power and worth. In all likelihood, humankind has always wished to transcend reality and pursued eternal youth and the desire to be stronger, fitter or more handsome. The 20th century saw a societal revolution, the emergence of fashion and make-up at affordable prices, hormone treatments and aesthetic surgery combined to democratize the change in our physical appearance. Yet, intellectual capabilities and more complex phenotypes continued to lie well outside the scope of skilled make-up, steroid hormones and the scalpel.

Today, the emergence of CRISPR-Cas9 gene editing with its enormous potential for the precise manipulation of the genome places phenotypic tinkering at our fingertips. Suddenly, transhumanism's dreams may well be feasible or, at least, possible in the near future. However, genetic enhancement should not be confused with therapy. Gene-editing therapy is concerned with the treatment or cure of a disease or a genetic condition by changing the genetic information to ameliorate the patient's symptoms or even reverse the pathological effects of a disease. Genetic enhancement, on the other hand, is concerned with "improving" ourselves or our children so as to provide them with qualities that were not originally encoded in their genome. This represents a definite shift in concept and one that raises a raft of bioethical issues. Here, I identify just some of the questions that arise from this very real possibility of applying biotechnological techniques for our genetic enhancement. Should gene-editing techniques only be used in the therapeutic treatment of severe, incapacitating diseases? Should we allow people to enhance their personal characteristics (for instance, having perfect pitch, leaner or stronger muscles, blue eyes, a higher IQ, infrared sight, or being nearly impervious to physical pain)? Whether employed in therapy or enhancement, should gene editing be restricted to somatic cells (in born individuals) or should we allow embryos to be modified (the latter being especially relevant in inherited rare diseases affecting multiple organs)? The latter implies the modification of the germline and, hence, the genome transmitted to the progeny. Effectively, we would be changing the allele frequencies of future humans, which may well be akin to shaping the future of humankind.

Some bioethicists are opposed to the genetic modification of the germline or, at least, plead for caution, while others argue that, as parents, we should seek the best genome possible for our children, especially if the tools and the knowledge exist, and that to do otherwise would be irresponsible of us. However, international polls show that not everyone accepts gene editing for enhancement. In Europe, some countries are in favor of therapy but see genetic improvement as an abomination, whereas others (e.g. Spain) are in favor of both types of genetic manipulation. Meanwhile, a number of scientists are already drawing up wish lists of genes and genetic variants that encode specific phenotypic traits that could be marketed for gene editing future humans. But, here, we should not forget the irresponsible, precipitous genetic modification of at least three babies conducted in China by He Jiankui, who sought to create humans beings resistant to infection by the HIV virus, and ended up mutating human beings that will be genetic mosaics carrying induced mutations all their lives.

There is a thin gray line between therapy and enhancement, with no single position having been embraced to date by scientists, clinicians, bioethicists, or jurists. Moreover, the laws and recommendations regulating gene editing around the world are far from homogeneous. Yet, this is clearly a highly germane bioethical issue that will impact our future and which we, as a society, need to reflect very carefully upon from a multidisciplinary perspective.

Gene therapy and synthetic biology: life as software

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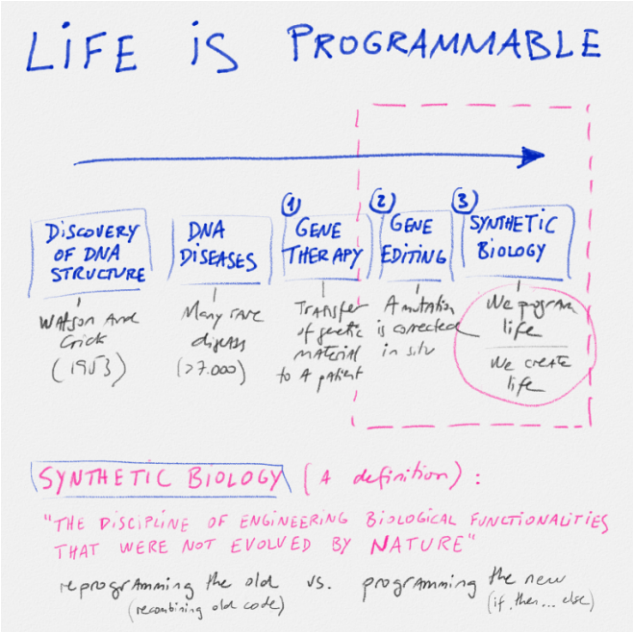
4th May 2020

Until very recently, the boundaries of biology were set by nature. But this might well be coming to an end: the emergence of the fields of gene editing and synthetic biology mean cells no longer have to play by nature's rules. SynBio, a combination of biology, genetics, programming and engineering, seems destined to revolutionize the life sciences and medicine. But at what cost? Are there any limits that we should impose on ourselves when it comes to editing cells?

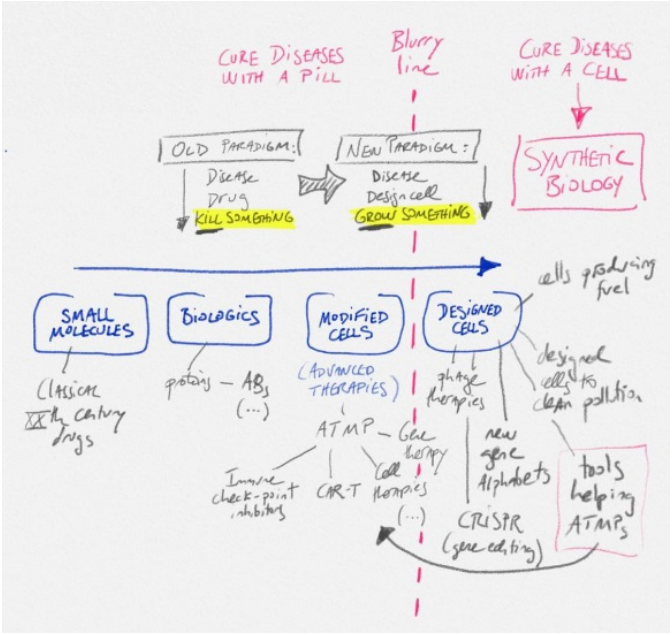
Since the late twentieth century, scientists have identified DNA as the "software" of living systems. Specialists are able to transfer parts of genetic code from one organism to another in order to "program" cells with specific functions. By means of DNA programming, SynBio seeks the use of artificial genetic codes to generate new behaviors in natural biology, or the artificial assembly of natural biological systems to generate new behaviors in living beings not designed by nature.

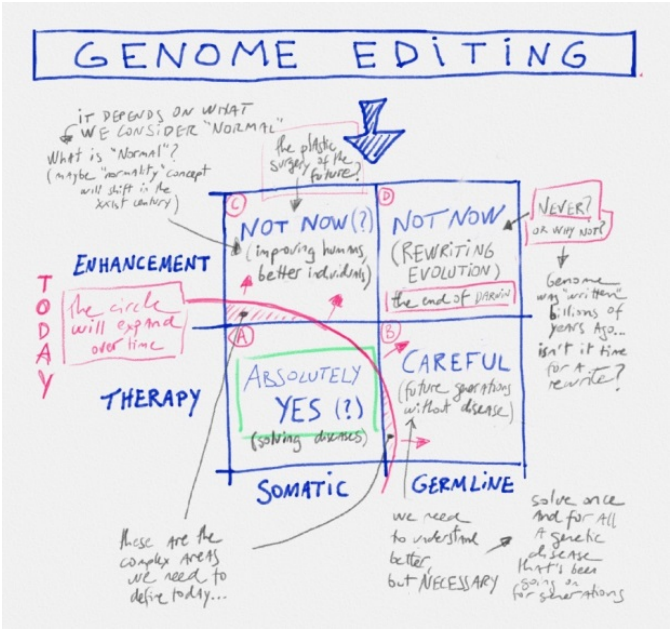


What has happened in medicine over the last century can be summed up as the evolution "from pills to cells". The first drugs were *small molecules*, simple substances that could tamper with biological pathways to remedy disease (e.g. aspirin and antibiotics). These simple drugs applied a paradigm that said (a) "there's something wrong", (b) "I administer a drug" and (c) "I kill something". Later, pharma evolved to produce biologicals, bigger and more complex proteins that were more precise and effective in tampering with those pathways causing disease (e.g. antibodies and insulin). By the end of the 20th century, the industry entered the stage in which it started supplying *modified cells* (e.g. CAR-Ts, gene therapy and advanced therapies), thereby shifting the paradigm to (a) "there's something wrong", (b) "I supply a cell" and (c) "I grow something", which today dominates the modern conception of medicine. The biopharmaceutical industry is entering a new era in which laboratories are able to provide newly *designed cells* (that is, cells built with a purpose, cells that previously did not exist in nature) to prevent and treat diseases.

We are on the verge of a Cambrian explosion of new applications. We have gone beyond simply reading and editing genomes to actually writing new genomes, creating our own ideas of what life should be like. The life code is four billion years old. It is time we began (with extreme care) to rewrite it. From the investor's perspective, there is always a point in time when a new technology changes from being "interesting" to being "investable". That time is now. Engineering living organisms is set to become one of the largest industries over the next few decades. The question, of course, is how far should we go? Are we

in danger of becoming too powerful? See my personal matrix below for debating the ethical issues of gene therapy.





The ethics of genome editing

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4th May 2020

The ethics of genome editing can be addressed from two points of view: a teleological perspective or a deontological perspective.

Genome editing from a teleological point of view:

Teleological ethics focuses on the consequences of acts or, in this case, of the technology being developed. Consequentialism (or utilitarianism) is its maximum exponent and analyzes the benefits of and the risks posed by this new technology. The problem with this perspective is that, in new technologies such as the one we are dealing with, we do not know what benefits or risks to expect. Faced with this situation, we might decide to act with caution and stop research altogether, thus avoiding the risks but also losing any potential benefits.

New genome-editing techniques such as CRISPR-Cas9 have led to an effective decrease in the methodological risks of the technique, since it has greatly increased the specificity, efficacy and versatility of gene editing while significantly reducing undesirable side effects and decreasing the difficulty of its use, making it accessible to many labs around the world. These features have made genome editing, which until recently was considered mere academic speculation, a more than plausible reality.

From a utilitarian point of view, a very cautious approach to the modification of wild species has been proposed given the serious environmental consequences such techniques might have (Ledford, 2015). As for their use in human species, positions differ depending on whether the modification is made in adults or to the germline. In the case of the former, there is almost unanimous acceptance if the genomic edition has a therapeutic goal; yet, opinions change radically when it comes to germline genome editing. The fact that, in this case, the genomic modification is passed on to offspring is both an advantage insofar as it eliminates the need to modify individuals as adults in the future and, at the same time, the main fear associated with the technique. Many different opinions have been expressed on this matter (for a review see Santaló, 2017), but the most widely accepted view is that genome editing should be allowed in basic research and only when the technology is sufficiently consolidated should it be used for therapeutic purposes, leaving genetic enhancement of the human species in a moratorium *sine die* (Santaló & Casado, 2016).

Genome editing from a deontological point of view:

In contrast, the deontological perspective bases its bioethical analysis on empirical reasoning, considering what is intrinsically good or bad. Here, the basic argument against genome editing is respect for genomic integrity. This argument holds that genetic modification violates the dignity of modified organisms (be they human, animal, plant or microorganisms) as it violates the genetic integrity of individual organisms.

This respect for genetic integrity can be considered not only at the individual level but also at the level of the species; thus, we should reject any genetic modification in any species — including the human species — regardless of the purpose of that modification. This approach is clearly aligned with the position of conservationism. Yet, the genetic modification of animal and plant species has been practiced by humanity for millennia in, for example, the selection processes made during domestication and the development of agriculture. However, it is argued that these processes are based on the random occurrence of mutations that are subsequently selected and that this is no justification for accepting a technology that is, clearly, not based on the introduction of random but rather of designed mutations.

The main proponent of this point of view has been Hans Jonas (1903-1993), who, in an essay entitled "The Imperative of Responsibility", wrote, "Act so that the effects of your actions are compatible with the permanence of genuine human life" (Jonas, 1984). However, Allen Buchanan argues that Jonas' proposal seems to assume axiomatically that the human species, as we know it today, represents the culmination of evolution and that any change in it must be worse, so we should make sure to preserve it as we know it. Such an idea seems to be radically contrary to the concept of biological evolution, which implies the idea of constant change. Whether this change is due to chance or is directed by an intelligent will (which is also the result of the evolution of the human species) may make a difference as to what is ethically acceptable or not, but it could also call into question the argument itself.

Multiple arguments have been forwarded from the deontological point of view, including respect for individual autonomy, eugenics and designer babies, equity and distributive justice, unsustainability and the naturalistic fallacy (for a review see Santaló, 2019).

Regardless of the point of view, genome-editing techniques have recently become the focus of ethical debate, particularly in the wake of news from China confirming the genome editing of two newborns in an effort to make them immune to HIV (Jiankui, 2018). This event has set alarm bells ringing and prompted a re-evaluation of technology control strategies (including proposals for moratoria) which, because of the characteristics of the technique, have been deemed unworkable.

The ethical debate is both relevant and essential. Society must participate in a decision that will affect the future of the human species, while postponing it or leaving it in the hands of a small number of individuals is likely to have irreparable consequences for everyone (Luna *et al.*, 2019). This debate, however, must be firmly based on evidence, accurate information and the avoidance of preconceptions; otherwise, its conclusions will be skewed and more likely to respond to individual interests than to the well-being of humanity.

Bibliography:

Buchanan A. (2011). "Beyond Humanity? The Ethics of Biomedical Enhancement". Oxford Scholarship Online doi: 10.1093/acprof:oso/9780199587810.001.0001

Jiankui, He, Human Embryo Editing Session. Second International Summit on Human Genome Editing, Hong Kong, 27-29 November 2018. Retrieved July 22, 2019, from: https://www.nap.edu/read/25343/chapter/1

Jonas H. (1984). The Imperative of Responsibility. In search of an ethics for the technological age. Chicago: Chicago University Press. USA. ISBN: 9780226405971. Translation of *Das Prinzip Veranwortung: Versuch einer Ethik fur die technolische Zivilization.* (1979).

Ledford H. 2015. Caution urged over editing DNA in wildlife (intentionally or not). *Nature:* 524(7563), 16. doi: 10.1038/524016a.

Luna F., Casado M., Turner S., Santaló J. 2019. Genome editing in humans, a topic only for academics from industrialized countries? *Revista de Derecho y Genoma Humano. Genética, Biotecnología y Medicina Avanzada:* 51, 43-60.

Santaló J. and Casado M. (coordinadors). 2016. Document sobre bioètica i edició genòmica en humans. Observatori de Bioètica i Dret de la Universitat de Barcelona. Publicacions de la UB. ISBN 978-84-475-4063-1

Santaló J. (2017). Edición genómica. La hora de la reflexión. *Revista de Bioética y Derecho*: 40, 157-165.

Santaló J. (2019). La mejora genética humana en los tiempos del CRISPR/Cas9. *Revista de Bioética y Derecho*: 47, 33-43.

Genetic editing in the public arena: communication and social perception

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Topics given prominence in public information influence the subjective establishment of the social agenda (what a specific community of individuals considers important at any given time), while the way in which information

is provided impacts perceptions of and public opinions about the problems or concepts in question (social representations of a reality).

Human genetic editing is one of the topics to have emerged in the public arena in recent years, especially since the expansion in the use of the CRISPR-Cas9 technique. It was precisely for this reason, that the Centre for Studies in Science, Communication and Society at the Pompeu Fabra University (CCS-UPF), in collaboration with the Vila Casas Foundation, dedicated the 2017 Quiral Report to a study of the communication of genetic editing and its social perception (1). The report describes how this topic was picked up and highlighted by the media and how its increased presence coincided with a surge in the number of Google searches from devices located in Spain, illustrating the agenda effect. The study also notes that the predominant metaphor for alluding to this technology was the image of "cut-and-paste", a metaphor that originated in scientific publications and which was then transferred to the popular media. It is critical that we reflect on the impact that a metaphor can have on an audience's perception of a concept, especially when they are unfamiliar with it. For example, using the metaphor of "cut-and-paste" is not the same as speaking of "molecular scissors", "genetic scalpel", "turn-on/turn-off", "techniques to create designer babies" or "Model T of gene editing", other metaphors employed in reference to this technology. Clearly, each metaphor is associated with its own world of referents: "cut-and-paste" conjures up images of digital word processors and is free of any especially positive or negative connotations, "scalpel" is associated with precision, while "Model T", a reference to the Ford Model T, is associated with something affordable and inexpensive, etc.

Genome editing, including the CRISPR-Cas9 technique, has also been the subject of ethical debates both within and outside the scientific community, especially when considering its potential use in the clinical context. CCS-UPF participated in the European Neuroenhancement and Responsible Research and Innovation (NERRI) project along with researchers from 11 other countries to examine public views and perceptions of genetic editing in the specific field of cognitive capabilities (2). In this project, four hypothetical scenarios were considered in which respondents were asked to make decisions about the use of technology to improve cognitive capabilities in healthy (neuroenhancement) and sick (neuro-treatment) individuals, adults and embryos. In all countries, a prevailing attitude of greater concern was detected towards the use of technology in embryos than in adults, especially when the aim was improvement as opposed to therapy. The specific applications of the technique, rather than the technology itself, seem to guide public opinion as to whether to accept or reject such interventions, although ethical debates tend to focus more on the technology.

It seems critical that we explore in greater depth just how genetic editing advances are communicated to the public and what views and attitudes the

public holds, not only in order to improve the communication of gene-editing techniques but also to guide decision-making involving the introduction of the technology and the ethical framework in which it should be developed.

Bibliography:

Revuelta G., Santamaria M., Ramírez A., Barbosa L., Gonzalo C., Armengou C. *Informe Quiral 2017*: La edición genética ante la sociedad. (2018) Fundación Vila Casas, Barcelona. Accessible at https://www.fundaciovilacasas.com/es/informe-quiral.

Gaskell, G., Bard, I., Allansdottir, A., Vieira da Cunha, R., Eduard, P., Hampel, J., Hildt, E., Hormaier, C., Kronberger, N., Laursen, S., Meijknecht, A., Nordal, S., Quintanilha, A., Revuelta, G., Saladié, N., Sándor, J., Borlindo Santos, J., Seyringer, S., Singh, I., Somsen, H., Toonders, W., Torgersen, H., Torre, V., Varju, M., & Zwart, H. (2017). Public views on gene editing and its uses. *Nature Biotechnology*, 35(11). https://doi.org/10.1038/nbt.3958


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