the Pontifical Academy of Sciences

New Development in Genome Engineering: Scientific and Ethical Aspects

Nicole M. Le Douarin

Professeur honoraire au Collège de France

Technologies for manipulating DNA have enabled advances in biology ever since the discovery of the DNA double helix in 1953.

An important step in the process that led to modern molecular biology and genetic engineering took place in the 1970s, when researchers realized that they could *use bacterial enzymes*, which evolved to defend bacteria against pathogens, to modify DNA in other organisms.

Two technologies for manipulating DNA that have yielded very significant advances in this field have to be mentioned: the first one makes it possible to cut the DNA molecule at specific sites and relies upon the discovery of the *restriction enzymes* that are used by bacteria to defend against viruses and plasmids. These enzymes recognize definite base sequences in the DNA molecule and produce very specific cleavage fragments. They permitted the development of the *recombinant DNA technology* that transformed molecular biology and medicine. The discovery of restriction enzymes was acknowledged by the attribution of the Nobel Prize, in 1978, to Werner Arber, Hamilton Smith and Daniel Nathan.

Another remarkable progress in DNA technology was the *Polymerase Chain Reaction (PCR)*, a technique that allows the amplification of a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. This technique was made possible by the isolation of a DNA polymerase from bacteria *Thermus aquaticus,* living in hot springs, that retains its activity at high temperatures, designated as the Taq enzyme. This major technical advance was acknowledged by the Nobel Prize in Chemistry to Kary Mullis and Michael Smith in 1993.

But one technology consisting in introducing site-specific modifications in the genomes of cells and organisms remained elusive. However, two techniques were developed, based on the principles of DNA-protein recognition. One uses site-directed *zinc finger nucleases* (ZFNs) and the other, called TALEN, requires another type of nucleases, the “Transcription activator-like (TAL) effector nucleases”. Difficulties of protein design, synthesis, and validation remained an obstacle to widespread adoption of these engineered nucleases for routine.

As David Baltimore said in his opening address at the International summit on *Human Gene Editing* that was held on December 1-3, 2015, at Washington, D.C., “*The methods available until recently were cumbersome and imperfect. Extending them to human beings was unthinkable. Over the years, however, the unthinkable has become conceivable”.*

This was three years after the beginning of a new era. In 2012 a new tool made possible the editing of the genome of any type of living organism, including human, in a way that cumulates so many advantages when compared to the pre-existing techniques, that it has revolutionized the world of genome editing.

This new tool has generated great excitement in the scientific and medical community because of its potential to advance biological understanding, to alter the genome of microbes, plants, and animal and to treat human diseases. It is designated by the following acronym: *CRISPR/Cas9* or *Clustered, Regularly, Interspaced, Short, Palindromic Repeats* and *CRISPR associated protein 9*.

Its name is certainly known by most of the people in this audience, but I thought that the story of this discovery could be of interest, for the non-biologists essentially, but also for all of us, because of the ethical questions that are raised by this technology.

The CRISPR/Cas9 system was devised by the laboratories of two women scientists: one is a French bacteriologist, Emmanuelle Charpentier, who received her scientific education at the Pasteur Institute in Paris and was working in Sweden at the time of the publication of the technique in 2012, and the other is American, Jennifer Doudna, whose laboratory is at Berkeley University.

What is the origin of the CRISPR/Cas9 system?

Before the CRISPR/Cas9 System was engineered by Charpentier and Doudna, significant work had been carried out on bacteria that paved the way to this remarkable achievement. This system, like restriction enzymes and PCR, relies upon the means developed by the bacteria to defend against viruses.

The CRISPR/Cas9 system has two components, both derived from the bacteria *Streptococcus pyogenes*: Cas9, the endonuclease enzyme able to cause double strand breaks in DNA, since it possesses two catalytic sites, and a guide RNA (sgRNA) which guides Cas9 to the location in the genome of the targeted sequence. This second component of the CRISPR/Cas9 system (or sgRNA) has two parts: one (of 20 nucleotides) that recognizes (by Watson-Crick complementarity) the site where the breaks are to be created in the targeted DNA, and the other being linked to a CRISPR-RNA (designated crRNA) necessary to bind and stabilize the Cas9 protein.

These single guide RNA molecules can be produced with much less effort and expense than for all the other techniques used before for the same purpose. The CRISPR system has been discovered, in several steps, as an adaptive immunity mechanism in bacteria.

In 1987 the Japanese bacteriologist Atsuo Nakata discovered in the genome of *Escherichia coli (E.coli)*, short repetitive sequences of the four constitutive bases that characterize the oligonucleotides forming the double strands of the nucleic acid sequence (i.e. of DNA or RNA: Adenine -A, Thymine -T, Guanine -G, Cytosine -C – replaced by Uracyle in RNA).

These repetitive sequences are distributed in palindromes (a palindrome is formed a sequence followed by the same sequence in the inverse order) that can be read in both senses and produce hairpin structures. *Between these repeated structures, short strands of DNA* are *interspersed*. This discovery failed to arouse the interest of the scientific community at that time. In 2002, they were designated CRISPR, but their role was still unknown. In 2004 bioinformaticians discovered that the DNA intercalated between these palindromic repeats was often pieces of DNA belonging to viruses that infected the bacteria.

In 2007 researchers working for a Danish firm producing yogurts found that, among the bacteria (*Streptococus thermophiles*) that they used to produce the yogurts, those containing viral sequences in the CRISPR regions of their DNA were able to resist viral infection much better than the ones that did not.

This observation was interpreted as the capacity of the bacteria to capture pieces of viral DNA in their CRISPR system to use them during a second infection to kill the virus. In the same way as the immune system of vertebrates keeps memory cells from a primo infection to fight against the same germ a second time. *This is the principle of vaccination*. The Vertebrate immune system is endowed with *adaptive* immunity because it is capable of *adaptation,* a process that increases its efficiency in fighting against an invader.

The discovery of adaptive defence mechanisms in bacteria was in itself an important discovery because it showed that organisms as simple and apparently as rudimentary as prokaryotes have developed a sophisticated type of adaptive immunity. Moreover, it is this bacterial system that has been adapted to produce a tool that made it possible to edit the genome of all types of organisms.

Using the CRISPR/Cas9 system as a tool to edit the genome: a success story

In contrast to ZFNs and TALENs, which require substantial protein engineering for each DNA target site to be modified, the CRISPR-Cas9 system only requires a change in the guide RNA sequence. For this reason, technology using the *S. pyogenes* system has been rapidly and widely adopted by the scientific community to *target, edit, or modify* the genomes of a vast array of cells and organisms.

Variations have been devised in the use of the CRISPR/Cas9 system. For example, the Cas9 enzyme can be mutated, to break the scissors, so that it still binds DNA at the site that matched its guide RNA, but no longer slices it. Instead, the enzyme stalls there and blocks other proteins from transcribing that DNA into RNA. Thus the system is hacked and the gene is turned off, without altering the DNA sequence. By inventing a few other tweaks, researchers have built a way to turn genes on and off at will. By fusion of a fluorescent protein (such as green fluorescent protein) to the Cas9 enzyme, they can also produce live-cell imaging of chromosomal loci.

Many laboratories around the world have used CRISPR-Cas9 to edit genomes of a wide range of cell types and organisms. Thousands of articles have been published that include the CRISPR acronym in the title or abstract, since the beginning of 2013.

CRISPR-Cas9 has a large array of possible applications. It provides a robust technology for studying genomic rearrangements and the development and progression of cancers or other diseases**.** Other examples are the systematic analysis of gene functions in mammalian cells *and* the use of CRISPR-Cas9 for genome-wide studies that will enable large-scale screening for drug targets and thus will expand the nature and utility of genetic screens in human and other cell types and organisms. Another important CRISPR-Cas9 application

with relevance to human health includes the ability to correct genetic mutations responsible for inherited disorders.

Ethical problems raised by the CRISPR/Cas9 system

In 2015 CRISPR/Cas9 was used in human pre-implantation embryos in China. The researchers used 86 tripronuclear zygotes (oocytes fertilized with two spermatozoa) that are not able to develop. The target gene was the##-globin gene that is mutated in #-thalassemia. They found that the endogenous gene was effectively cleaved (52%, 28 embryos) but many *off target events* were recorded and the embryos were mosaics with cells having different genomes. They found high rates of repair using endogenous sequences. These were obvious obstacles to gene therapy strategies using CRISPR/Cas9, since they could lead to unwanted mutations. This result showed that more work was required for using this system on human material and this first experiment was at the origin of serious ethical concern about the possible use of this technology in human reproduction.

In December 2015 scientists of major world academies called for a moratorium on inheritable human genome edits that would be passed on in pregnancies, including those related to CRISPR-Cas9 technologies, *but supported continued basic research and gene editing that would not affect future generations*. This position was further adopted by other Academies such as in England where British scientists were given permission by regulators to genetically modify human embryos by using CRISPR-Cas9 and related techniques, on condition that the embryos were destroyed in seven days. In the US the National Academies of Sciences, Engineering, and Medicine released a report on their “Recommendations for Responsible Conduct” of gene drives.

In genetics, “gene drive” means that the inheritance of a gene or a set of genes can be positively biased, even if it circumvents the Mendel traditional rules. In the case of a specific gene that normally has a 50-50 chance of being passed along the next generation, a gene drive, that can be achieved through CRISPR/Cas9, could push the rate to nearly 100%.

Several laboratories have shown that gene drive works in practice in the laboratory in fruit flies, mosquitos and yeast. Its application in the wild could be envisioned, for example, to fight malaria transmission. An anti-malaria gene drive might change a mosquito’s genome so that the insect no longer had the ability to accept the malaria parasite, thus stopping the spread of the disease. A gene drive that forced all offspring to be male would make reproduction impossible, thus wiping out an entire population of a given species.

Opponents of the technique fear that permanently altering life forms on Earth will impact species and ecosystems in a way likely to be irreversible. As an example of the reactions raised by gene drive technology, I mention that the British primatologist Jane Goodall and dozens of other environmentalists and scientists have signed an open letter that calls for a halt to all proposals for the use of gene drive technologies “*given the obvious dangers of irretrievably releasing genocidal genes into the natural world*”.