FACULTAD DE MEDICINA
UNIVERSIDAD DE CANTABRIA

GRADO EN MEDICINA

TRABAJO FIN DE GRADO

La tecnología del CRISPR/CAS9 como una herramienta para la investigación y tratamiento del cáncer

The CRISPR/CAS9 technology as a tool for cancer research and therapy.

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ABSTRACT

CRISPR-Cas9 is a novel gene editing tool that came from the immune system of bacteria against viruses, modified to become one of the most efficient technologies in molecular medicine. The main goal of this final degree project is to systematically review the state-of-the-art about the CRISPR-Cas9-based gene-editing tools and their applications in cancer therapy and investigation. A total of 142 articles were used, with any type of design published from 2010 to 2021 with a 3.0 or higher Journal Impact Factor. In investigation, the CRISPR-Cas9-based technology can form knock-outs, knock-ins and chromosomal rearrangements mimicking carcinogenesis and, additionally, can be used to screen for these modifications with CRISPR-Cas9 libraries. Most of its potential is focused towards therapy, with clinical trials on-the-go, being most of the efforts directed to correct the inactivation of tumor-suppressor-genes, to delete fusion oncogenes and to counteract viral proteins in viral driven cancers. In immunotherapy, it contributes to the programming of CAR-T cells and the inhibition of immune checkpoints. Finally, CRISPR-Cas9 can screen for mutations responsible for drug resistance, helping to establish an alternative to poorly responsive tumors. Even though the risk of off-target mutations is the main disadvantage, CRISPR-Cas9 gives hope for high precision medicine.

***KEY WORDS*:** CRISPR-Cas9; Gene editing; mutations; therapy; cancer

RESUMEN

CRISPR-Cas9 es una nueva herramienta de edición de genes derivada del sistema inmune de las bacterias contra los virus, modificada para establecerse como una de las más eficientes estrategias disponibles en la medicina molecular. El objetivo principal de este trabajo de fin de grado es recordar los mecanismos de reparación del ADN y revisar sistemáticamente el funcionamiento del CRISPR-Cas9 y sus aplicaciones en terapia e investigación del cáncer. Se usaron 142 artículos publicados desde 2010 hasta 2021 incluyendo todo tipo de diseño, con un Factor de Impacto superior a 3.0. En investigación, CRISPR-Cas9 puede eliminar genes (knock-outs), inserción de genes (knock-ins) y reordenar cromosomas en modelos carcinogénicos, aparte de su utilidad para rastrear mutaciones con genotecas específicas. En terapia, su uso se ha dirigido al desarrollo de ensayos clínicos, encaminados fundamentalmente a restablecer genes supresores de tumores, suprimir fusiones oncogénicas o inactivar proteínas tumorales virales. En inmunoterapia, ayuda a programar las células CAR-T e inhibir los puntos de control. Finalmente, CRISPR-Cas9 puede detectar mutaciones implicadas en la resistencia a agentes quimioterápicos, constituyéndose una alternativa en tumores de mal pronóstico. Aunque su mayor desventaja sean las mutaciones generadas fuera del sitio diana, CRISPR-Cas9 ofrece esperanzas hacia una medicina de alta precisión.

PALABRAS CLAVE: CRISPR-Cas9; edición genes; mutaciones; terapia; cáncer

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ABREVIATIONS

AAV: Adeno-Associated virus

Acr: anti-CRISPR

ALK: Anaplastic lymphoma kinase

APC: adenomatous polyposis coli

ATR: Ataxia telangiectasia and Rad3-related protein

BARD: BRCA-associated RING domain 1

BART: BamHI A rightward transcript

BRCA1: Breast cancer Susceptibility gene 1

BRCA2: Breast cancer Susceptibility gene 1

CAR T cells: chimeric antigen receptor T

CCDC67: Coiled-Coil Domain-Containing Protein 67

CGH: Comparative Genomic Hybridization

CRC: colorectal cancer

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

*cr*RNA: CRISPR RNA

CTLA-4: cytotoxic-T-lymphocyte-associated protein 4

CUL3: Cullin 3

dCas9: dead Cas9

DDR: DNA damage response

dGTP: deoxyguanosine triphosphate

DGK: diacylglycerol kinase

DNA-Pk: DNA dependent kinase

EBNA: EBV nuclear antigen 1

EBV: Epstein Barr virus

eGFP: Enhanced Green Fluorescent Protein

EGFR: epidermal growth factor receptor

ELP5: Elongator complex subunit 5

EML4: Echinoderm microtubule-associated protein-like 4

ERK: extracellular signal regulated kinase

EWSR1: EWS RNA Binding Protein 1

FER: Feline Encephalitis Virus-Related Kinase

FISH: Fluorescence in situ hybridization

FL1: Friend leukemia integration 1 transcription factor

GVHD: graft-versus-host disease

HPV: human papilloma virus

KLF11: Kruppel-like factor 11

KRAS: Kirsten rat sarcoma viral oncogene homolog

LAG-3: lymphocyte activation gene-3

LMP1: latent membrane protein 1

MAN2A1: Mannosidase Alpha Class 2A Member

MED12: Mediator of RNA polymerase II transcription

MEK: mitogen-activated protein kinase

MHC: Major histocompatibility complex

MLL1: methyltranferase 1

NCTP: sodium taurocholate co-transporting polypeptide

NF1: Neurofibromatosis type 1

NF2: Neurofibromatosis type 2

NK: natural killer

NSCLC: non-small cell lung cancer

P53BER: p53 bound enhancer regions

PAMs: Protospacer Adjacent Motifs

PD-1: Programmed Death 1

PD-L1: Programmed death ligand 1

RAMPs: Repeat-associated mysterious proteins

Rb: retinoblastoma gene/protein

RNP: ribonucleotide proteins

Rpn13: 19S proteasome-associated ubiquitin receptor

S212/S312: Aurora A Kinase to phosphorylate P53

SCID: combined immunodeficiency disease

SDF-1: Chemokine stromal cell-derived factor-1

*sg*RNA: single guide RNA

shRNA: short hairspin RNAs

siRNA: small interfering RNA

SMAD4: Mothers against decapentaplegic homolog 4

TADA1: Transcriptional Adaptor 1

TADA2B Transcriptional Adaptor 2B

TCEAL1: Transcription Elongation Factor A Like 1

TCR: T cell receptor

TGF-β: transforming growth factor β

TKI: tyrosine kinase inhibitors

TMEM135: Transmembrane Protein 135

TRAC: TCRα constant

*tracr*RNA: trans-activating RNA

Treg: T cell regulator

*tr*RNA: transcription RNA

VHL: von Hippel Lindau gene

WNT: wingless-related integration site genes

1. INTRODUCTION

The CRISPR-CAS9 technology can be easily summarized in two words: genetic scissors. It is a tool for genome engineering with the ability to precisely cut in a specific area of our genes and with that, it is able to edit them. The CRISPR-CAS9-based gene-editing technology is one of the many tools discovered for this purpose, but its popularity and importance is justified by the very simple and effective mechanism that relays behind it, and its infinite types of applications in modern medicine have led the researchers responsible for its development to be awarded with the Nobel Prize in Chemistry in 2020.

In its origin, the CRISPR-CAS9 is a part of the immune system of bacteria – a rather surprising discovery since bacteria are commonly understood as very simple organisms characterized by a very primitive cellular system compared to eucaryotic cells. Nonetheless, a Spanish researcher, Francis Mojica, from the University of Alicante realized back in 1997 that not only bacteria but also archaea have a region in their genome composed of several regularly spaced repetitions that nowadays it is termed as Clustered Regularly Interspaced Short Palindromic Repeats or CRISPR. Mojica suspected that in these organisms with limited complexity, the need to destine a part of their genes for these sequences of repetitions had to have a significative importance, especially in survival, since the presence in archaea demonstrates an evidence of ancestry (1).

The role of CRISPR in bacteria is based on incorporating a short sequence of the virus DNA that has infected them to their genome with a cluster of short DNA repeats separated by 'spacer' sequences. This part of the genome containing the virus DNA is transcribed as RNA, and therefore is recognized by a trans-activating RNA (*tracr*RNA) that fits perfectly with the spacer sequences. This allows it to be linked by Cas9 proteins, that cut this RNA in small pieces that contain parts of the DNA virus. As shown in Figure 1, once the bacteria is reinfected by an identical virus, it is able to recognize the same sequence previously incorporated in their genome, and cleave the virus's DNA, protecting the bacteria from it (2)(3).

The wide range of possibilities to use this tool in research started a race to publish this discovery. And the idea to use this as a gene editing tool was what allowed Emmanuelle Charpentier and Jennifer Doudna to step in front of this race and eventually be conferred as the 2020 Chemistry Nobel Prize winners.

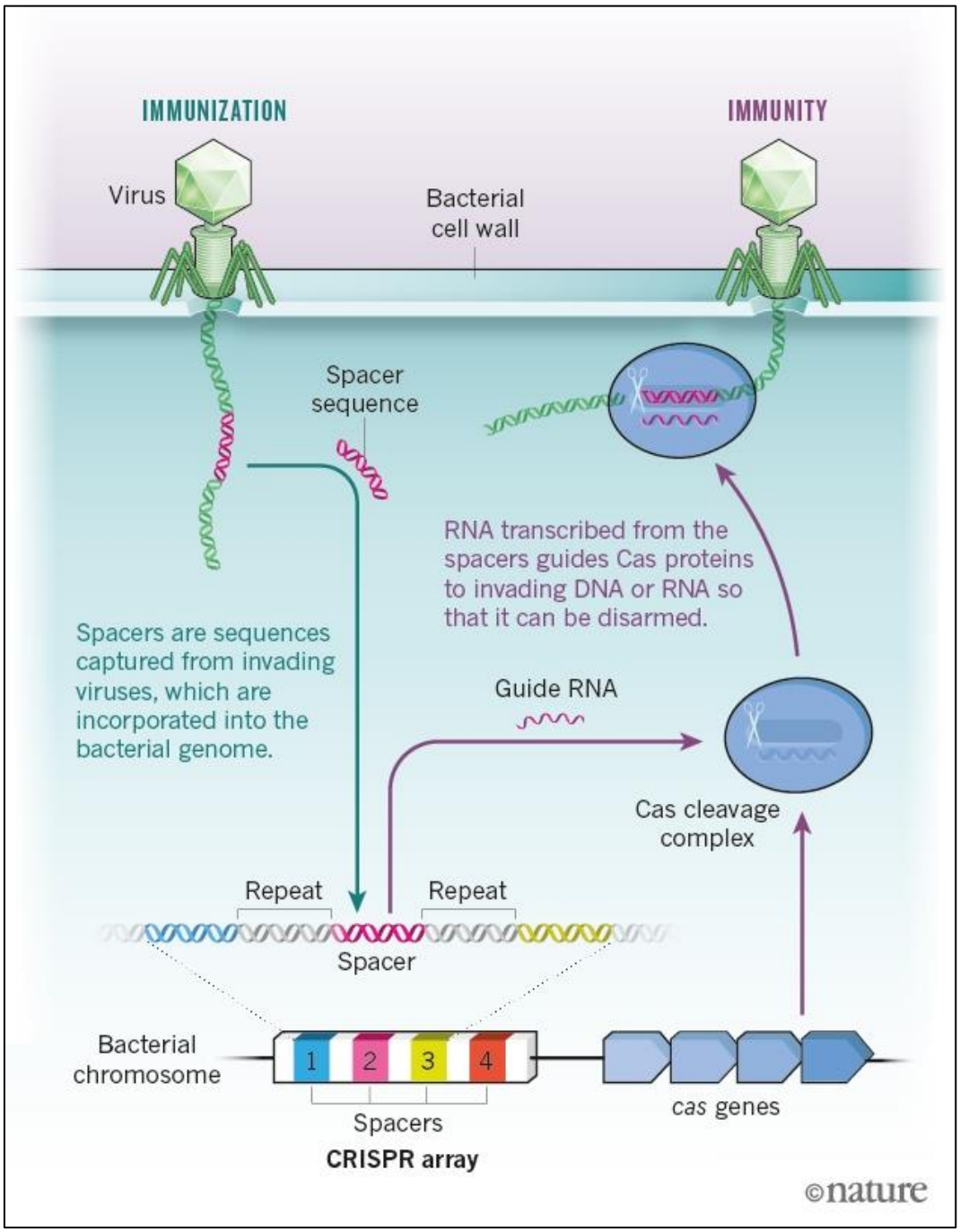

Figure 1. The CRISPR-Cas9 Mechanism. (Adapted from Nature - https://www.nature.com/news/five-big-mysteries-about-crispr-s-origins-1.21294)

Emmanuelle Charpentier had spent years studying the gene regulation of *Streptococcus pyogenes* whilst Jennifer Doudna work was focused on RNA interference. Their paths aligned when they realized that the bacteria's immune system functioned as a RNA interference. Charpentier was the one scientists responsible for finding the *tracr*RNA previously mentioned and needed the help of a biochemist such as Doudna to further boost her research on this topic. Once working together, their main goal was to make this mechanism as simple as possible – they decided to fuse the tracrRNA and CRISPR-RNA in to one molecule that, since then, will come to be known as guide RNA or single guide RNA (sgRNA) (4).

When a cut in our DNA occurs, the human cell has very effective repair mechanisms that are triggered, which can generate two different outcomes: in the first place, the gene that was cleaved can be inactivated, creating what is known as a knock-out. In second place, if a DNA template of a pre-established sequence is inserted in the zone that was cut, it can introduce an entire new gene, creating a knock-in (Figure 2).

A third possibility, also based in using a DNA template, the system can repair the gene that was cut, or edit it, correcting a wrong placed nucleotide (3). Before the

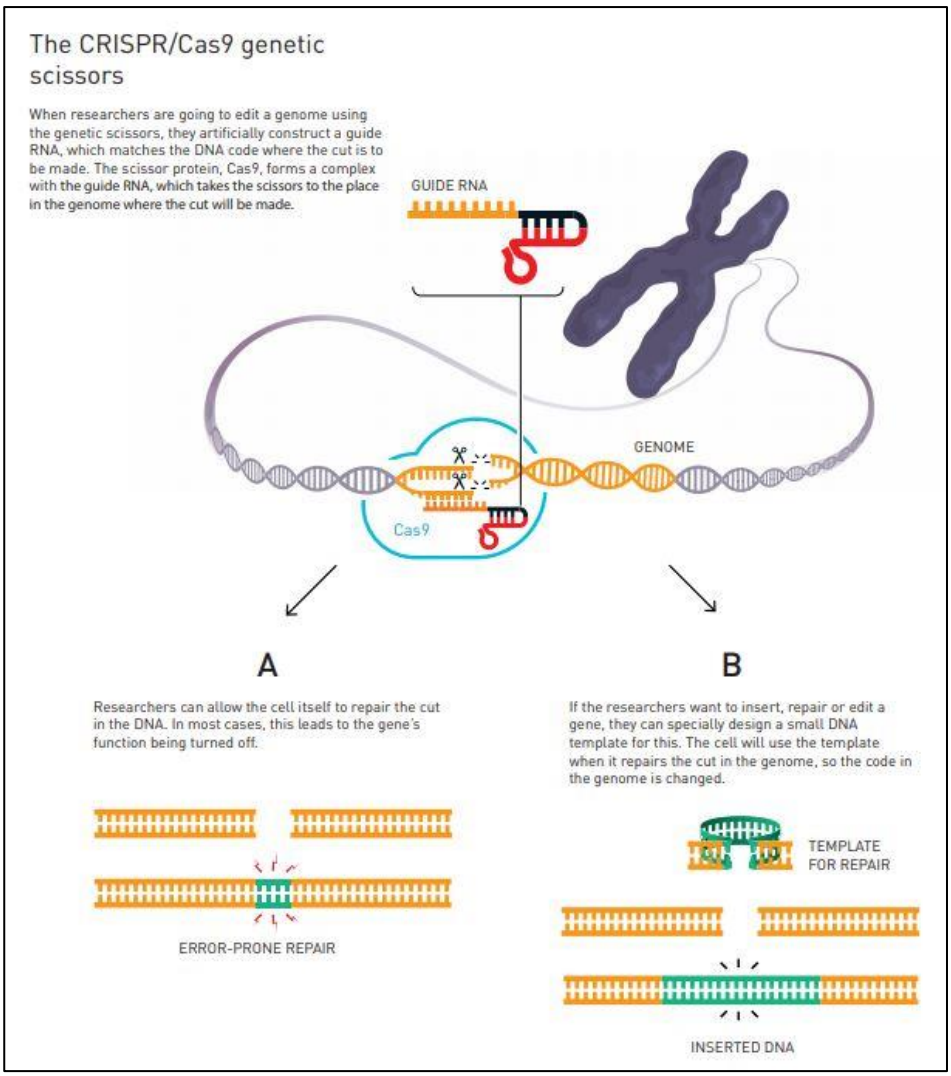

Figure 2. The CRISPR-Cas9 genetic scissors. This image describes the way the CRISPR-Cas9 technology can be used as a genetic scissors (Modified from nobelprize.com).

development of the CRISPR-CAS9-based gene-editing tool, the precise control of the many steps that are required in this process was a difficult task for biotechnology. Therefore, the advantage of this form of DNA editing is the very little complexity needed to design a particle that can recognize any sequence we desire and cut it as intended with a very high precision: the direct and indirect implications of this discovery are unmeasurable to life sciences and medicine. Some of the applications will be described below, with a wide range of different approaches in genetic diseases: from monogenic illnesses such as sickle cell disease and polygenic and epigenetic pathogenesis, like cancer.

Regarding to monogenic diseases, there are several clinical trials currently underway using this technology to cure sickle cell disease (NCT03745287) and β-Thalassemia (NCT03655678, NCT03728322) (5–7). Since they are hematological diseases, cells are easier to manipulate *ex vivo*. Excitingly, some preliminary results have been positive, since two patients with β-Thalassemia and another one with sickle cell disease no longer needed transfusions (8). As a consequence, other monogenic diseases have been considered good candidates to be treated with CRISPR-Cas9 technology, such as Leber congenital amaurosis 10 (9). Moreover, there is also a clinical study for transplantation of CRISPR CCR5 modified CD34+ cells in HIV patients (NCT03164135) (10).

On the other hand, in cancer, the CRISPR-CAS9-based gene-editing technology has the ability to edit human genome to restore tumor suppressor genes (such as *TP53*) that have been inactivated at some stage during carcinogenesis and to disable genes that thrive cancer progression. This concept may seem very easy to understand, but the technology that was available before CRISPR-CAS9 for this purpose was very time-consuming, difficult to work with, which translated to a very distant future of applying genome editing to everyday medical practice.

With this new technology it is possible to reproduce *in vitro* and *in vivo* models in investigation to try to recreate cancer mutagenesis, progression and survival and, therefore, providing a new approach to unreveal molecular mechanisms involved in carcinogenesis that may serve as new targets for chemo- and immunotherapy. This research models are based in three different strategies: generating knock-out genes, recreating chromosomal rearrangements and gene correction by introducing knock-ins, as a way to try to discover their implications in the cell's functions and how that ends up in developing a tumor that is able to survive infinitely and to grow and spread to affect other tissues. They can be also used to recreate a complex environment similar to the one behind tumorigenesis by combining different kinds of gene editing: for example, we can design CRISPR CAS9 to create loss-of-function of the adenomatous polyposis coli (APC) gene (a tumor suppressor gene related to gastrointestinal cancer) or to promote proliferation by adding genes such as KRAS that induce cell growth and stimulate mitosis (11).

Furthermore, because of the similar cellular pathways implied in viral cancers, CRISPR CAS9 is able to inactivate oncogenes (E6, E7) that have been induced by viruses such as human papillomavirus (HPV), provoking cell apoptosis and suppressing the

development of cancer linked to HPV (12). This mechanism might be analogous to many other types of virus driven cancers and it is a very promising area of investigation.

Moreover, the upcoming trend of chimeric antigen receptor T (CAR T) cells for immunotherapy was also furthered by the CRISPR CAS9 technology. The strategy is based on obtaining a blood sample from a cancer patient in order to reprogram the lymphocytes T *in vitro* to fight against their own tumor and then reintroducing the new tumor specific T cells back to the patient. It is in the *in vitro* manipulation that CRISPR CAS9 proves to be useful, since it can be used to generate the T cell antigen receptor and to adapt it to recognize specific antigens expressed by the ongoing tumor.

Last but not least, it has been largely documented that chemotherapy has limited effects in inducing remission in several cancers due to the development of drug resistance tumors as a consequence of mutagenesis and expression of new resistance genes. CRISPR CAS9 can be used to suppress those genes that make the oncogenic cells not sensible to former chemotherapy drugs.

The wide range of applications for CRISPR CAS9 and its attractiveness for lab sciences around the world demands an obligation to raise an ethical questioning: which are the barriers of this technology? Genome editing is widely discussed since the first studies showed up the possibility of editing embryos and even grown humans and this simple and effective way of editing genes brings a new concern to the scientific community. Besides that, the employment of this mechanism in clinical trials also stands an ethical obstacle to overcome. Nevertheless, this tool was able to revolutionize the expectations of genetic therapy as it was conceived before and brings very high expectations to its future uses in medicine.

2. OBJECTIVES

The availability of an easy and specific method for gene editing has always been an interesting but unattainable approach for cancer therapy and investigation. However, it seems that CRISPR-Cas9 came to revolutionize this field. With this technology, real models for tumorigenesis both *in vitro* and *in vivo*, can be generated and new targets for chemo- and immunotherapy can be found. The main goal of this final degree project review is to compile the information about the uses of the CRISPR CAS9 technology in cancer, specifically in cancer research and therapy.

More specifically, the main goals for this project are: to review the state-of-the-art of the CRISPR CAS9 applications explaining how it can be used as a gene editor. Next, we will remember the cell's repair systems as a way to better understand the usage of this tool and to elucidate how the editing of genes with CRISPR CAS9 is a way to recreate tumorigenesis and therefore can be used as an instrument to comprehend better the pathogenesis behind cancer. Another objective of this work is to explain the uses of CRISPR CAS9 in the field of immunotherapy: the search of new targets, to manipulate and correct checkpoint alterations and as a technology that it is useful to facilitate the generation of CAR-T cells. Last but not least, we will dedicate a section to describe the value of CRISPR CAS9 in viral driven cancers and to portray the usages of this technology for chemotherapy resistance.

3. METHODS

This is a systematic review of articles depicting the applications of the CRISPR-Cas9 technology in cancer research and therapy. The inclusion criteria were articles published from 2010 to 2021 consulting PubMed, National Center of Biotechnology Information, OMIM and GeneCards databases, written in English, Spanish or Portuguese. No restrictions were made in spite of the study design, including experimental and observational studies. The papers considered for this project had more than a 3.0 Journal Impact Factor, using the InCites Journal Citation Reports (*https://jcr.clarivate.com/JCRLandingPageAction.action*). A total of 142 articles were selected and therefore will be cited in this final degree project.

4. HOW OUR CELLS REPAIR THEIR DAMAGED DNA

To better understand the CRISPR-Cas9 mechanism of action, we will first review the cell's repair mechanisms in order to construct a better comprehension of the subject matter.

The DNA is formed by **nucleotides** that contain a nitrogenated base, a five-carbon sugar (deoxyribose) and a phosphate group. The different types of nucleotides are cytidine (C), uridine (U), adenosine (A), guanosine (G) and thymidine (T). When the phosphate is removed, the resultant molecule is called a **nucleoside**. (13) The different nucleotides bind to each other in the same strand by phosphodiester unions that are covalent bonds, and with other nucleotides in the complementary strand following base pairing rules (adenosine to thymine and cytosine to guanosine) with hydrogen bonds.

The DNA of an eucaryotic cell is damaged daily by thousands of different mechanisms that can be grouped in two sets: those generated by the cell itself and the other ones as a product of external alterations. The first class is produced by the very active cell environment that generates heat and metabolic incidents that can harm the DNA. Among these mutations, we can include replication errors, spontaneous base deamination, abasic sites, oxidative damage and DNA methylation. The second class can be generated by radiations (UV or ionizing), biological agents (bacteria's toxins and viral proteins) or chemical agents: alkylating agents, aromatic amines (such as the polycyclic aromatic hydrocarbons found in tobacco) affecting the DNA sequence directly or indirectly (14).

The consequence of these continuous attacks to the cell's genes throughout the evolution has been the development of a very powerful and complex repair system whose main goal is to maintain the DNA sequences as unaltered as possible. The importance of this repair system is very easily understood if we think of how many cancers bear mutations leading to diminished levels of components of the cell's repair structure, such as Breast cancer Susceptibility gene 1 and 2 (BRCA-1 and BRCA-2) related to breast and ovarian cancer, Xeroderma Pigmentosum gene associated with skin cancer and mutS (E. coli) homolog 2 (MSH2) related to colon cancer.

The eukaryotic repair mechanisms can be classified in two groups: those able to repair single strand alterations and those able to repair double strand alterations (15).

4.1. MECHANISMS OF SINGLE-STRAND BREAK REPAIR

The design of the double strand DNA is what enables the cell's repair systems to be so effective and precise: when a single strand mutation occurs, the other non-damaged strand is available to be used as a template for the reparation, leaving little space for errors.

Single strand modifications include two different types of local mutations: base mutations and nucleotide mutations. Each one has its own repair mechanism described below:

A) Base mutations and base excision repair

Base mutations imply the change of one nitrogen base once the DNA is replicated, being commonly produced by deamination or depurination that occur spontaneously in the cell. This type of modifications to the DNA can be repaired by base excision mechanisms.

The base excision mechanism consists of a **DNA glycosilase** that has the capability to search in the DNA for alterations using a base-flipping method, that allows this enzyme to cover the entire surface of the nucleotides to detect mutations. Once a base mutation is encountered, the enzyme removes the selected base from the sugar, creating a gap in the DNA (Steps A-C in figure 3). The gap is recognized by **an apurinic or apyrimidinic endonuclease** that cuts the phosphodiester bond. Afterwards, the gap is refilled with the correct analogous base, using the other non-harmed strand as template by the **DNA polymerase** (Step D in Figure 3)

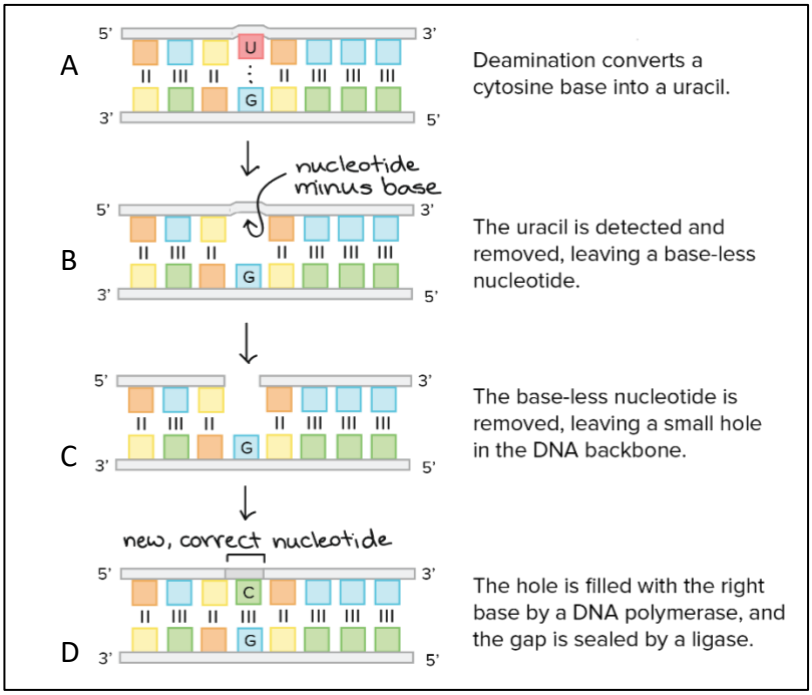

Figure 3. The base excision mechanism. The mechanism of base excision is described in this figure, step by step (Adapted from Khan Academy).

B) Nucleotide mutations and Nucleotide excision repair

The nucleotide mutations are more complex and extended when compared to base mutations, being usually a product of external cell damage such as tobacco or radiation.

A common DNA damage is the one produced by UV radiation that creates a bond within two adjacent pyrimidines, forming a **pyrimidine dimer** that makes it impossible to replicate the DNA (Step A in Figure 4). The mechanism to repair this kind of mutation is similar to the one explained before but using it in a larger scale that includes entire nucleotides. The pyrimidine dimer is detected, an **excision nuclease** eliminates a short sequence of nucleotides including the altered ones that is accompanying the mutation, the **DNA helicase** separates the excised nucleotides from the other strand and in the following step, the **DNA polymerase** synthesizes a DNA sequence analogous to the non-damaged strand (Steps B-C in Figure 4). Finally, the **DNA ligase** glues the new sequence to the preexistent one, repairing the damage (Step D in Figure 4). The step that separates the two strands is regulated by Xeroderma pigmentosum (XP) family of ATP- dependent helicases, the XPB and XPD. The absence of these proteins produce a diseased named like the proteins, where the exposure to UV lighting correlates to a much higher risk of developing skin cancer (16).

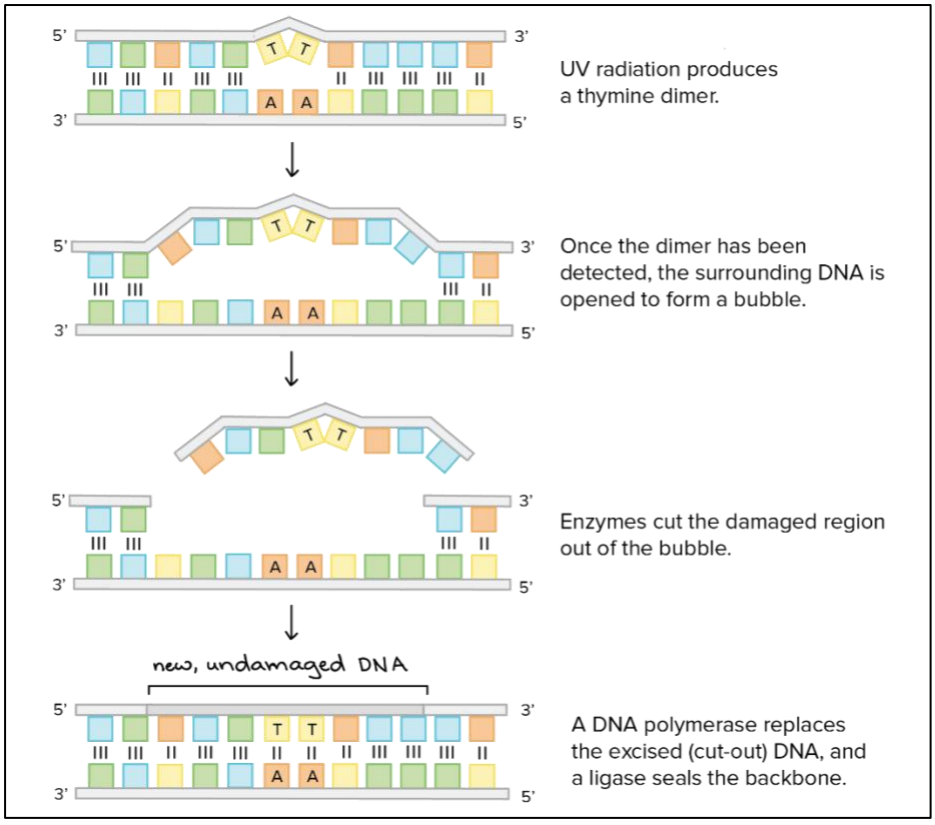

Figure 4. The mechanism of repairing the pyramidic dimers formed by UV lights. (Adapted from Khan Academy).

4.2. MECHANISMS OF DOUBLE-STRAND BREAK REPAIR

Double strand alterations include injuries produced by ionizing radiation and oxidizing agents that can compromise the double helix structure. However, most frequently, they are produced by internal damaging during the formation of replication forks that become broken. The difficulty to repair this type of mutations is explained because there is no template available to use in comparison to the alterations mentioned in the previous section. This explains why these repair instruments are not so accurate, and therefore, less efficient when rivalled to the single strand's one. There are two different ways to repair double strand mutations:

A) Nonhomologous end joining

When a cut of the DNA affecting the double helix is produced, the nucleotides near the break usually are damaged and can't be repaired, so they are excised of the sequence. The exonucleases that are involved in this step are **WRN**, deficient in Werner syndrome; and **Artemis**, absent int human severe combined immunodeficiency disease (SCID). Both of these nucleases are phosphorylated **by DNA dependent kinase** (DNA-PK); whose functions are described in the next section. This highlights the importance of the correct catalyzation of this stage.

The remaining nucleotides are linked together by **DNA ligase**, joining the two ends of the strand together. The joining of the two ends is facilitated by DNA-PK, by a process involving the formation of a complex that bridges the two ends and help enlist the variety of proteins involved in the assembly, such as DNA ligase. The direct consequence of the malfunctioning of this cascade of proteins is defective DSB repair, radiation sensitivity and increased mutagenesis (17).

This form of repairing is not a very precise mechanism, nevertheless, humans have a very large proportion of DNA that is not constantly activated, so in overall it does not produce important alterations to the cell's functioning. On the other hand, sometimes, when the two ends of the strands are connected, they create a fusion of chromosomes thus provoking chromosomal aberrations that can difficult appropriate cell division (15).

B) Homologous recombination

This way of repairing the double strand breaks (DBS) is only available short after DNA replication and before the cell is divided because they use the sister chromatids as templates. Just as it was explained before, the advantage of having templates is a more accurate reparation and hence, less errors. Moreover, this strategy is not only widely used by the cell to restore different kinds of mutations, but it is also the mechanism behind the chromosome recombination produced during meiosis that allows to create variability among the offspring.

The basis of the homologous recombination is the existence of several areas in human chromosomes that have extensive regions that are very similar to each other, what is called **homology**. And the easiest way to have homologous sequences near to each other is after the replication, because the sister chromatids naturally lye together, so the non-damaged chromatid can be used as a template for the damaged one.

More specifically, after the double strand break is created, nucleases introduce small cuts to produce exact 3' ends. These pieces of DNA with 3' ends are able to exit the damaged DNA and to insert theirselves in between the non-harmed DNA double strand, finding the exact homologous region that corresponds perfectly to the nucleotide sequence. This process is called **strand invasion** and it is regulated by **Rad51** (18).

This protein, Rad51, not only has this major role in DNA homologous recombination, but also can reinitiate RNA replication forks when damage is detected and promotes cell survival when there is genome instability (19). The importance of understanding its function in the cell's repair mechanisms relies on the fact that mutations in this protein can lead to tumorigenesis: when it is overexpressed, it can cause various types of mutations that can help proliferation and invasion; on the other hand, its absence has also been linked to tumor progression (20). Therefore, its accurate regulation is a crucial process in DNA repair.

Moreover, Bhattacharya et al. (2017) added a new function to Rad51 correlated to immunity. They were able to demonstrate that the downregulation of Rad51 triggered by itself the activation of the immune system, as a mechanism of marking DNA damage. Subsequently, the activated immune system promotes cell apoptosis and the exposition of cell's antigens – a response that favors autoimmunity (19).

When the homologous pair is created, the DNA polymerase copies the non-damaged sequence to introduce the missing nucleotides, repairing the alteration. Once this process is completed, the new repaired sequence goes back to the double strand it belonged, and the DNA ligase incorporates it to the previously existing chromosome (15).

C) TUMOR SUPRESSOR GENES IMPLICATED IN DOUBLE-STRAND BREAK REPAIR

→ *DNA damage response (DDR)*

When the cell is exposed to reactive species of oxygen (ROS) and a genotoxic environment, it triggers what is known as a DNA damage response (DDR), that is able to screen for DNA damage, correct it, arrest cycle-cell progression and induce apoptosis when all of the other strategies fail. The main effectors of this pathway are ATM (ataxia-telangiectasia mutated), ATR (ATM- and Rad3-Related) and DNA-PKs. (21). This is summarized in Figure 5.

ATM functions as the DNA damage scan, being regulated by the MRN complex (composed of Mre11, Rad50 and Nbs1) that acts as a double-strand break sensor and recruits ATM to broken DNA molecules. When MRN detects double strand breaks, it enhances its kinase activity in order to activate BRCA-1, Chk2 and p53 phosphorylation. The direct consequence of the activation of these proteins is the cell cycle arrest, accompanied by promotion of repair and apoptosis.

Moreover, DSB also activate ATR, which shows overlapping functions with ATM when this occurs. The difference, however, is that ATR can be also activated by a variety of other DNA damages. For example, in response to a replicative stress there is an overexpression of single stranded DNA (ssDNA) that is a consequence of stalled

replication. This activates ATR, which triggers a downstream cascade of phosphorylation of kinases, “cleans” the replication sites of the ssDNA, initiates cell checkpoints and can even induce apoptosis if the damage is beyond repair. The triggering of the cell checkpoints rely on the inhibition of CDC25 (22).

These two pathways also interrelate to each other: first, ATM can promote the activation of ATR and vice-versa and they can both bias the recruiting of each other to DNA damage. Likewise, they can also phosphorylate each other and can be functionally redundant to secure the DNA’s repair (21).

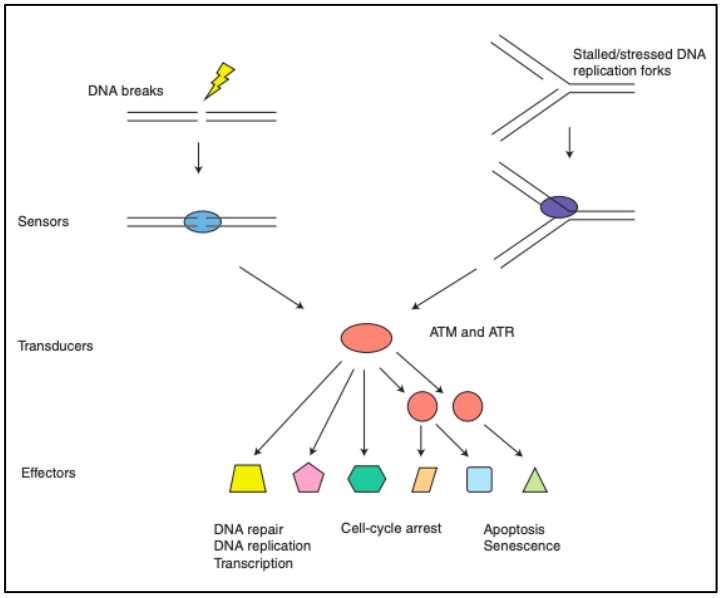

Figure 5. **Genes implicated in double-strand break repair.** The DNA breaks and stalled DNA replication forks are sensed and activate ATM and ATR that function as transducers. Therefore, they induce DNA repair and replication, cell-cycle arrest or apoptosis. (Adapted from Maréchal and Lou, 2015)

→ *Breast cancer Susceptibility gene 1 and 2 (BRCA-1 and BRCA-2)*

BRCA1 participates in different aspects of DNA repair. In the first place, it forms a heterodimer with BRCA-associated RING domain 1 (BARD) to create an E3 ubiquitin ligase activity in order to activate itself when DNA is damaged and participating in DDR. In the second place, it is part of the many proteins that set up the cell cycle checkpoints. Finally, it directly participates in HR and NHEJ. On the other hand, BRCA2 is responsible for recruiting Rad51 to homologous recombination in the repair of double strand breaks (16). These genes are widely known as the breast cancer genes, since they are the main genes mutated in hereditary breast cancer, being the life time risk of developing breast cancer in BRCA1 mutated women up to 70% compared to non-carriers (23).

5. CRISPR-Cas9 MECHANISMS

CRISPR stands for **C**lustered **R**egularly-**I**nterspaced **S**hort **P**alindromic **R**epeats and its structure is made by **short repeated sequences** of DNA that come up to 28-37 base pairs (bp), intercalated by spacers that have the same bp’s length. To better comprehend this technology, it is important to recall it is part of the immune system of the bacteria. In greater detail, the different parts that composes it are:

a) ***Short Repeated Palindromic Sequences***: as shown in Figure 6 when each repeated sequence is read in the direction 5'-3', it is identical to the complementary strand, even though they lie in opposite directions (one in 5'-3' and the complementary in 3'-5'). That's what is called a **palindromic repeat**. The importance of having palindromic sequences is the ability to form loops. The repeats are conserved from generation to generation (24). They are represented in Figure 7) as a diamond figure.

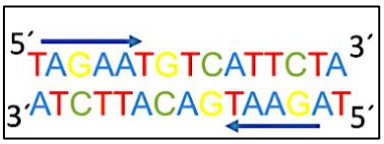

Figure 6. Example of a Palindromic sequence

b) ***Regularly Intercalated Spacers***: the palindromic repeats are intercalated regularly by what is called **spacers**. The spacers are a **genetic record** of all the viruses that have infected the bacteria. They can stock a large number of DNA sequences, varying from bacterial species (25).
For example, when a bacteria is attacked by a bacteriophage (a virus that infects bacteria), it activates the CRISPR immune system and introduces a part of the virus DNA into their own DNA, forming spacers. Therefore, the spacers serve as a strategy to have a "criminal record" (or a "genetic record" in this case) of the viruses, in order to recognize them again if they are reattacked. The process is analogous to the adaptative immune system in humans with memory B cells and the high precision is what differentiates it with other immune systems found in other bacteria or archaea.
In contrast with the repeated sequences, since they are a combination of the virus DNA, they are not conserved in each generation, changing depending on the different virus or plasmid that had invaded the bacteria (24). They correspond to the rectangles in Figure 7

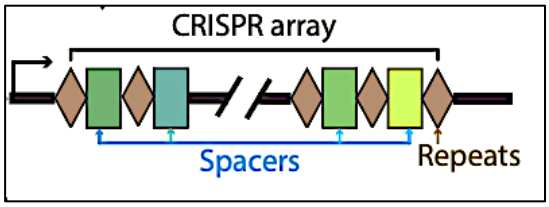

Figure 7. Structure of the CRISPR array. (Adapted from Jiang F., Doudna J.A ., 2017)

The loci also contains a leader sequence that functions as a promoter. It has a length of approximately 500bp and has a very significant role in transcription signaling (24). It is represented by the black arrow in Figure 7.

Next to the CRISPR loci there is the Cas gene site that is transcribed as Cas proteins. The Cas proteins have a role in every step of the bacteria's immunity attainment: they are needed to incorporate the invading virus DNA in the bacteria, they are involved in the production of the transcription RNA (*tr*RNA) and, finally, they also participate in the RNA interference phase (26). The steps of the immunity defense will be described in the next sections.

5.1. TYPES OF CRISPR CAS

First of all, it is necessary to stablish some main concepts that are going to be widely used throughout the chapter. When the CRISPR sequence is transcribed, the Cas genes are also included, generating a **transcription RNA** or ***tr*RNA**, in other words, they are all transcribed in one single RNA. When we are only describing the CRISPR part of the molecule, it is called **CRISPR-RNA** or **crRNA** and it doesn't include the cas genes.

The recombination of the CRISPR sequences with the different cas genes generates 6 different types of CRISPR-CAS, classified accordingly to the Cas protein they use. They can be grouped by classes whether they function with a multiprotein cleavage complex or a single-protein one (26). The classification is described in the Table 1 below (27).

	CLASS I			**CLASS II**		
	I	III	IV	II	V	VI
Cas type	Cas3	Cas10	Csf	Cas9	Cas9	C2c2
Effector complex	Multiprotein CRISPR RNA complexes			Single multi-domain protein		

Table 1. Classification of CRISPR-Cas systems

Not all types of systems are found in all bacteria, for example, the CRISPR-Cas9 is specially found in *Streptococcus pyogenes* and *Neisseria meningitides* (28). The cas1 and cas2 are ubiquitous of all bacteria. They play a role in the adaptation part of the immune system (29).

The **Type I** includes Cas3, a large protein with two distinctive domains: helicase and DNAse. In addition, it also has gene encoding proteins that participate in cascade-like reactions interplaying with Cas5 and Cas6 families. The participation of all of these domains are the way that Cas3 manipulates the crRNA, creating a transcript with long spacer repeats (28).

The **type II** system is the simplest of all the cas proteins because it has the lower number of genes involved. Since it has few genes, the proteins involved have to incorporate different domains with distinctive functions in order to work as a whole system. Thus, the cas9 protein has at least two nuclease domains that edit the genome sequence: a RuvC domain near the amino terminus and the HNH domain in the middle of the protein. The HNH has also an endonuclease activity, being the one responsible for target cleavage (28). Moreover, it also functions as a cluster sequence that is analogous to the ones present in the CRISPR – therefore, it is able to bind to the CRISPR RNA since it has a homologous sequence to it and form a complex. This will be better understood in the following point. The simplicity of the system made it suitable for using it as a gene editing tool.

Type III has been found in *Staphylococcus epidermidis* and include Cas10 and Cas6. Cas 6 has already been mentioned above, since it also works in the cascade activated by Type I. In contrast with the other two types, type III has a large participation of Repeat-associated mysterious proteins (RAMPs), that possess also RNAse activity, being involved in the processing of crRNA. The RAMPs can work in unison with Cas1-Cas2 or separately (28).

Types IV-VI have been recently described and their course of action is still not well understood. All the different types described are represented in Figure 8

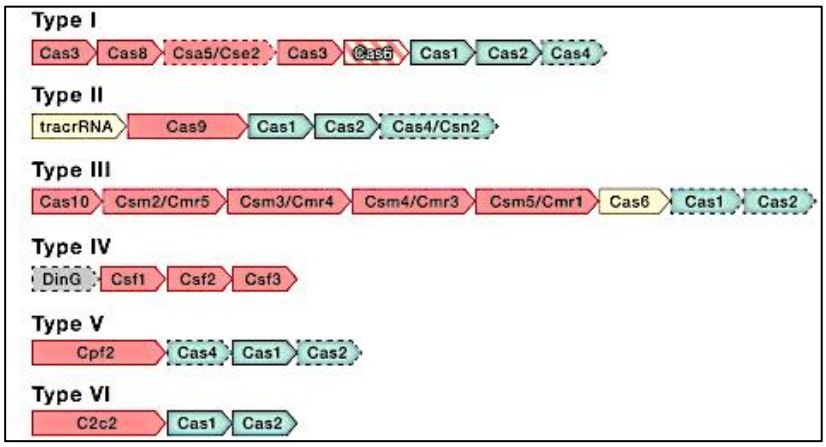

Figure 8. Types of CRISPR-Cas9. (Adapted from Wright et al., 2016)

5.2. A BACTERIAL DEFENSE MECHANISM: CRISPR CAS9

The CRISPR CAS9 defense mechanism is divided in three steps: 1) spacer acquisition or Adaptation, 2) CRISPR RNA (crRNA) genesis and 3) RNA interference (27). This is represented in Figure 9.

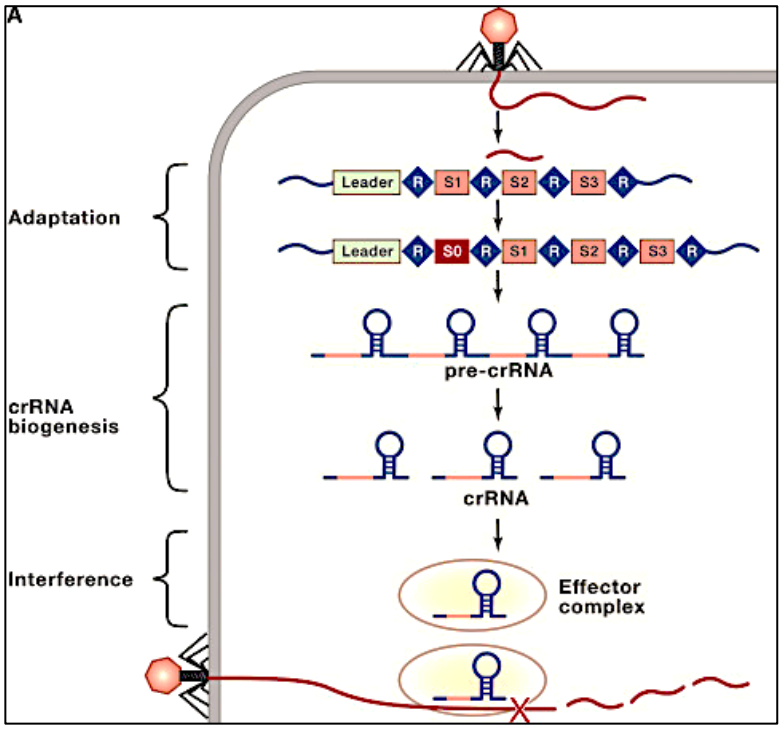

Figure 9. The three steps of CRISPR immune system: adaptation, crRNA biogenesis and interference. (Adapted from Wright et al., 2016)

→ *Spacer Acquisition or Adaptation*

This is the step in which the genetic material of the virus invading the bacteria is incorporated in to the CRISPR sequence. The proteins that are responsible for this phase are Cas1 and Cas2 that combine to form a heterodimer: **Cas1-Cas2 complex**.

When a bacteriophage attacks a bacteria, the viral DNA enters the host cell so it can insert itself into the DNA and uses the bacteria's replication system to replicate. When the DNA reaches the cytoplasm, the Cas1-Cas2 complex recognizes short motifs that are situated next to the target virus sequence, called **Protospacer Adjacent Motif**s (**PAMs**). Once Cas1-Cas2 bind to the PAMs, they use their cleavage domain to cut the target sequence generating a **protospacer DNA.** The Cas1-Cas2 complex then acts as an integrase, performing two transesterification reactions in the CRISPR array DNA. The 3'OH of each protospacer strand makes a nucleophilic attack on the repeated sequence of the CRISPR array, one at the leader side and one in the spacer side, incorporating into it. All of these steps are shown in Figure 10. Every time the bacteria is invaded by a phage, a new spacer sequence is incorporated to the CRISPR array in chronological order (26).

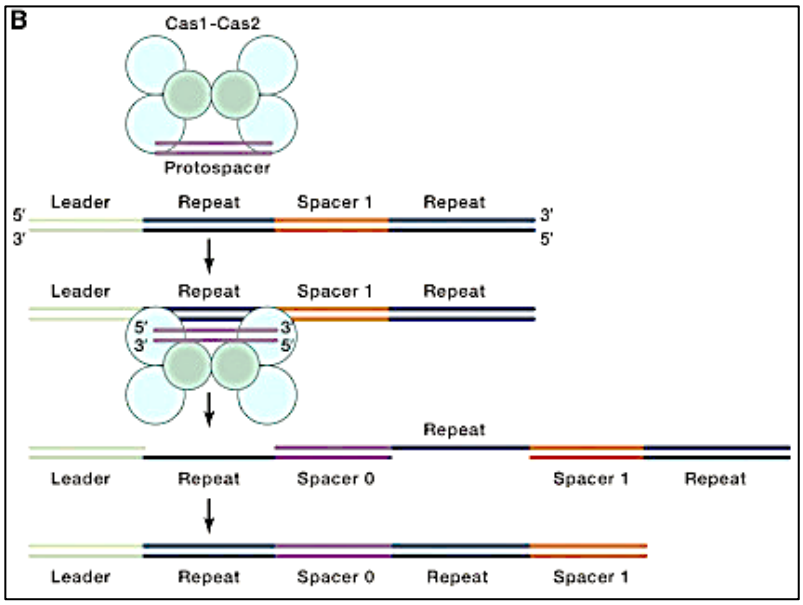

Figure 10. Formation of the Cas1-Cas2 complex and the generation of the protospacer DNA. (Adapted from Wright et al., 2016).

→ *Biogenesis of CRISPR RNA*

The CRISPR array is then transcribed to RNA in a tree-step procedure. The steps are described in Figure 12.

1- In the first part, it forms a CRISPR RNA precursor (**pre-crRNA**), that form loops due to the presence of the palindromic repeats, as represented in Figure 11.

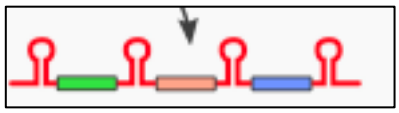

Figure 11. The formation of pre-crRNA. (Adapted from Burnistrz et al., 2020).

2- In the second step, the **tracrRNA** is also transcribed and unites itself to the pre-crRNA, helping the maturation of the structure. It has a hairpin structure, represented in green in Figure 12

3- In the third step, the pre-crRNA is then processed by **Cas9** (represented by the red circle in Figure 10), which with the help of **RNAse III** (represented by the orange triangle in Figure 12) separates the different protospacer regions included in the pre-crRNA with the corresponding flanking palindromic repeats. Finally, it forms a complex with the Cas9, being called the **Cas9-crRNA-tcRNA effector complex**. As a result, we have one protospacer for each Cas9-crRNA-tcRNA effector complex (26). In the example represented in Figure 12, the blue protospacer has been incorporated to the complex.

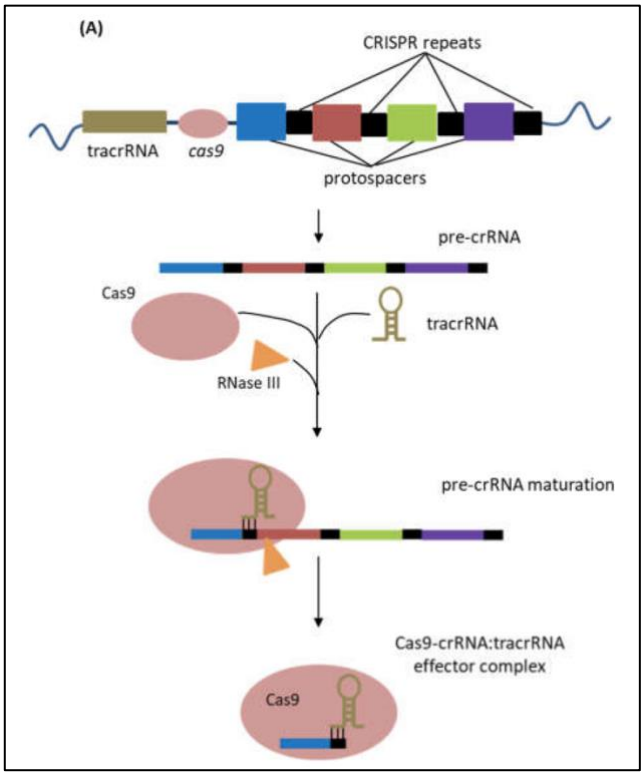

Figure 12. Assembling of the Cas9-crRNA-tcRNA effector complex. (Adapted from Montecillo et al., 2020).

→ *RNA interference*

When the bacteria is reinfected by the same phage, the Cas9-crRNA-tcRNA effector complex recognizes the PAMs, which are a short consensus sequence of three nucleotides: NGG, represented in Figure 13. Once the recognition is made, the complex binds to the virus DNA, allowing the Cas9 proteins to play their role of genetic scissors by creating double strand breaks in the target DNA. The ability to create double strand breaks is enabled by the two catalytic sites mentioned before: RuvC and HNH, each one

responsible for breaking a single brand (28). When the breaks are generated, the virus DNA is inactivated and therefore, the bacteria can escape from its invasion.

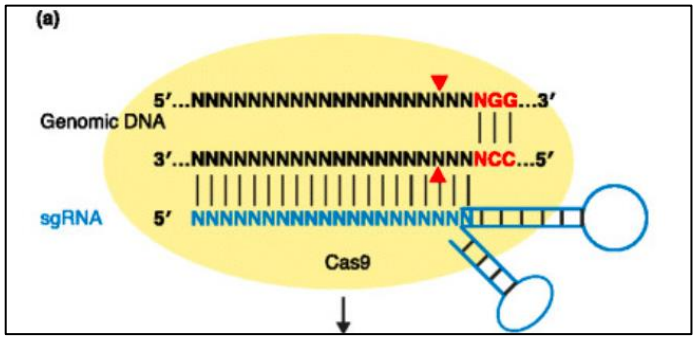

Figure 13. The protospacer adjacent motifs. Recognition of the PAMs: short consensus sequences of three nucleotides – NGG. (Adapted from Mou et al., 2015)

Jinek et al., realized that the dual crRNA and tracRNA can be engineered to a single RNA to simplify the process. For that purpose, they designed a chimeric RNA that contained a recognition sequence at the 5' end with a hairpin structure that mimicked the base-pairing interactions in between crRNA and tracRNA. This sequence is necessary for guiding the site-specific cleavage performed by Cas9 (30). This was the initial hallmark that opened the gate to the possibility of developing a new technique for genome editing (Figure 14).

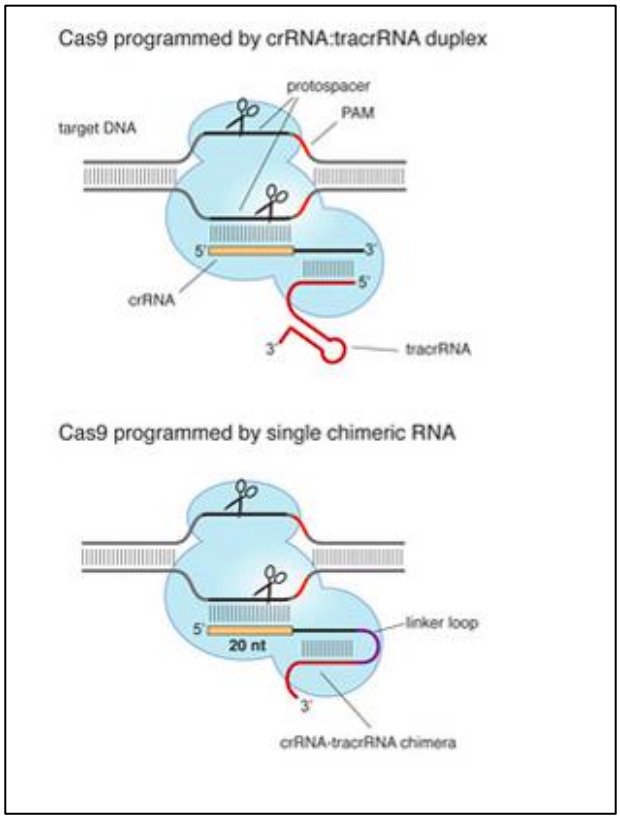

Figure 14. The design of a single chimeric RNA that functions as the crRNA:tracRNA complex. (Adapted from Jinek et al., 2012).

5.3. GENE EDITING

As introduced before, the chimeric unified RNA of crRNA:tracRNA makes the CRISPR CAS9 system very simple since it only has 3 components: the CRISPR sequences, cas9 proteins and a single guide RNA. By designing a CRISPR sequence that is analogous to a predeterminate one, the Cas9 is able to recognize it, binding to the target DNA and activating the catalyzing domains of Cas9 which generates a double-strand break in the DNA. As previously mentioned, double-strand breaks can be repaired in two ways, being the Non-Homologous reparation, the most common route followed by the damage provoked by CRISPR-Cas9. In spite of the mechanism of repair, the result of the activation of the repair systems can be used to create knock-ins, knock-outs, indel mutations and frame-shift mutations that can inactivate a whole gene (26).

Furthermore, since each catalytic site of Cas9 is responsible for breaking one strand, CRISPR-Cas9 can be designed to correct or to create point mutations. For that, a cytidine deaminase is added to the Cas9 lacking the cleavage domains, called dead Cas9 or dCas9. When the dCas9 binds to the target area, the cytidine deaminase changes the cytidine to an uracil. Because uracil is not found naturally in the DNA, the cell recognizes it promptly, changing it to a thymidine. When DNA replication is initiated, it identifies in that strand the newly introduced thymidine, and inserts in the new strand the homologous adenosine. Therefore, in a site where initially there was a C-G now it is replaced by an A-T (31).

In the other hand, not only the catalytic property of Cas9 has an importance in gene editing, since it was discovered that when the Cas9 binds to a target DNA without being able to cleave it can still inactivate the gene. Therefore, it functions as a transcription regulator, silencing genes when the Cas9 binds to them. In order to do that, the two catalytic sites, RuvC and HNH, are mutated, but the domain that recognizes the target sequence is not manipulated. Moreover, dCas9 can be united to an RNA polymerase, directing it to promotor regions where genes are poorly expressed. This new function defines Cas9 as a regulator, not only an inactivator (30).

The homology directed repair can produce gene correction, insertion, precise point mutations, gain-of-function and loss-of-function mutations. (26).

To summarize, the CRISPR-Cas9 gene editing tool permits double-strand breaks and single-strand breaks to be done, and also works as a gene promotor regulating transcription. This is represented in Figure 15.

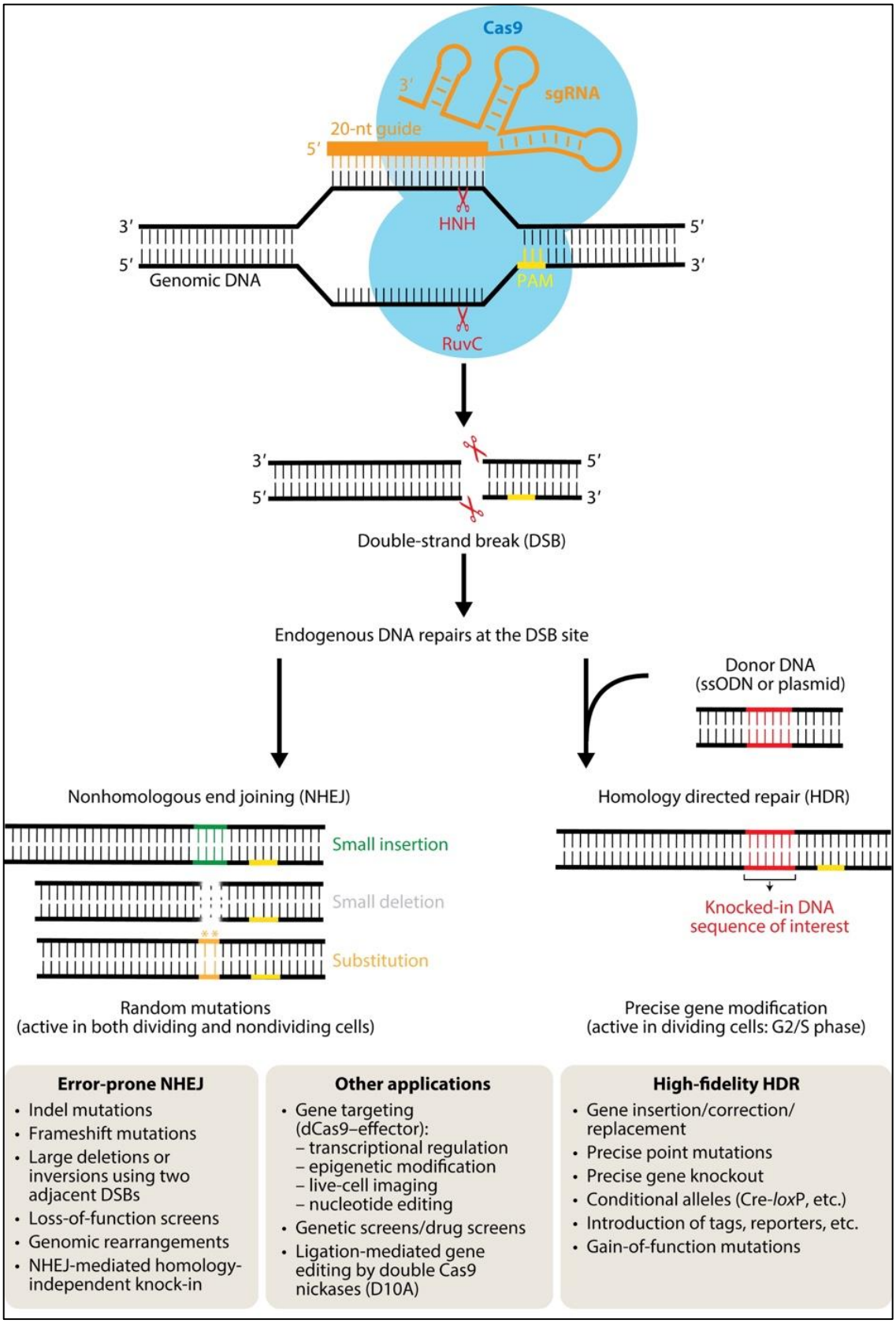

Figure 15. CRISPR-Cas9 as a gene editing tool: applications. This figure summarizes the functioning of CRISPR-Cas9. In the first place, the system cleaves the DNA with the two catalytic sites, HNH and RuvC, producing a DSB. That incites an endogenous repair, that can follow two pathways: the NHEJ or the HDR, depending on the presence or not of a donor DNA. (Adapted from Jiang F., Doudna J.A., 2017)

6. USE OF CRISPR-CAS9 IN CANCER RESEARCH AND INVESTIGATION

The initial application of the CRISPR-Cas9 technology was its use to recreate the molecular changes underlying cancer. Although nowadays tumorigenesis is better comprehended, there are still many steps to unreveal and a non-complex gene editing tool can boost this grasp forward. This will signify a better way to predict cancer behavior, to introduce new targets for treatment or to enhance the ones used until today. And, moreover, bringing a more specific approach to cancer treatment that directs itself precisely to the tumor cells and, consequently, it might potentially reduce the side effects that radio-, chemo- and immunotherapy provokes.

Three different ways of manipulating the genome can help to understand tumorigenesis: generation of knock-out genes, introduction of new genes (knock-in) and recreation of chromosomal rearrangements.

6.1. KNOCK-OUT GENES

It is known that tumorigenesis doesn't rely in one single mutation – it is a succession of cumulative mutations that collaborate to produce an ideal environment for proliferation and growth. With CRISPR-Cas9, this same environment can be mimicked by generating a serial sequence of knock-out genes. The best way to illustrate this application is compiling the information about very well studied tumors, such as colorectal cancer (CRC), where the process of tumorigeneses is well stablished as the **adenoma-carcinoma sequence**, with APC, TP53, KRAS and SMAD4 being the most common mutations described until today. For this experiment, two different groups were formed: a control group that didn't have the CRISPR-Cas9 system, and the experimental group that introduced this technology inducing different mutations (32). Before explaining the experiment, it is important to understand the participation of these mutations in CRC:

a) In a cell expressing the wild version of the APC gene, the β-catenin is phosphorylated by APC (which in turn is the final target of a cascade of kinases previously activated), inducing ubiquitination and consequently, proteosome degradation. The β-catenin is a protein that, when it reaches high concentrations in the cell, it is able to migrate to the nucleus acting as a transcription factor to induce the activation of the wingless-related integration site genes (WNT), involved in cell proliferation. Therefore, the inactivation of β-catenin as a consequence of the normal APC's function leads to suppression of proliferation (33)(34). Represented in Figure 16.

b) The KRAS pathway is activated by the epidermal growth factor (EGF) through its binding to the EGF receptor (EGFR) located in the cell's membrane. The activation of KRAS induces an enzyme cascade resulting in activation of BRAF, mitogen-activated protein kinase (MEK) and extracellular signal regulated kinase (ERK), which cooperate stimulating cell growth, proliferation, angiogenesis and migration – the 4 elements that sustain a tumor (35).

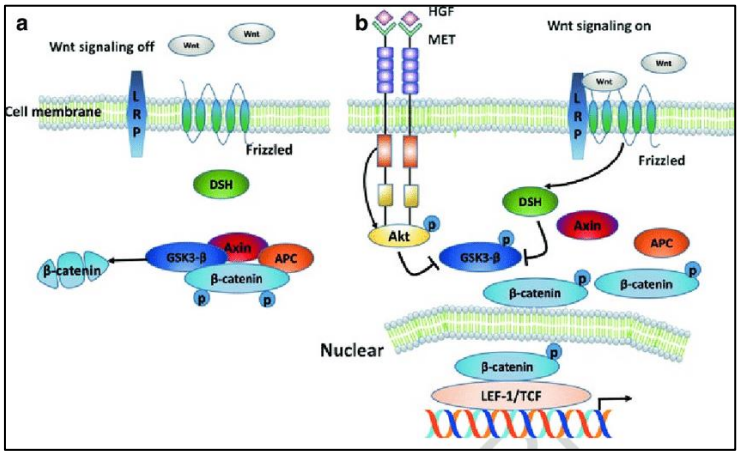

Figure 16. APC, WNT and β-catenin molecular pathways. (Adapted from Qian Xe et al., 2017).

c) SMAD4 also plays a role in tumor suppression, since it inactivates the transforming growth factor β (TGF-β) signaling pathway that induces cell growth and migration. Consequently, the loss of this gene is correlated with cancer (36).

d) The TP53 gene is known as the cell's guardian because it has the purpose of maintaining the correct functioning of the cell: when the DNA is damaged, p53 induces repair. And, when the repair is not possible, it stimulates p21 that provokes cell cycle arrest by inactivating checkpoint cyclins, thus leading to apoptosis (37).

All the steps mentioned above are summarized in Figure 17.

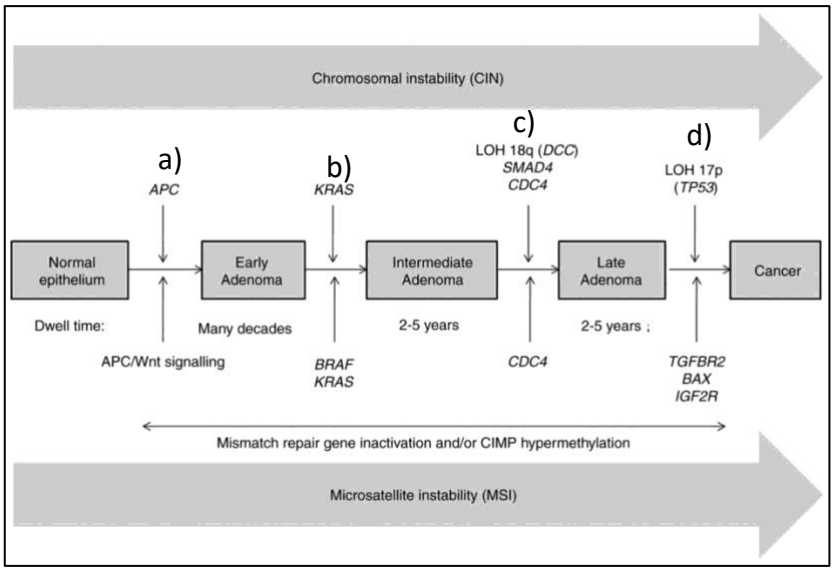

Figure 17. Transition from Adenoma to Carcinoma. (Adapted from Nguyen et al., 2018).

In the work of Drost et al. (2015), they used the CRISPR-Cas9 technology to first delete the APC gene, since it is well documented that this process is one of the first steps leading to CRC. Once this clonal culture with APC loss is obtained, again with the CRISPR-Cas9 technology, the TP53 gene was inactivated. As a result, there was a clonal expansion of the mutated cells. To generate the KRAS mutation, they designed an

oligonucleotide with the oncogenic mutation and two silent mutations that were used as a template for homologous recombination. And, finally, the SMAD4 mutation was crafted, activating the TGF-β pathway. They observed that, when the 4 mutations were engineered in the same cell, the power of this combination is such that environmental growth factors are not needed anymore for cell proliferation; subsequently the tumor that emerged as a consequence of this mutation sequence is totally independent *in vitro*. The *in vivo* introduction of these 4 mutations enables the formation of solid tumors masses that invade such as actual CRC. Moreover, only APC and TP53 mutations are required for the formation of aneuploidy (32).

Additionally, the CRISPR-Cas9 technology can be a tool for screening these mutations. With that purpose, a lentiviral vector was designed carrying the Cas9 and sgRNA simultaneously, added to a short hairspin RNA (shRNA). Afterwards, it was introduced to a melanoma model cell line resistant to vemurafenib – a monoclonal antibody directed to v-Raf murine sarcoma viral oncogene homolog B, (BRAF) mutations and found that the resistance is associated to loss of function of Neurofibromatosis type 1 (NF1), Mediator of RNA polymerase II transcription (MED12), Neurofibromatosis type 2 (NF2), Cullin 3 (CUL3), Transcriptional Adaptor 2B (TADA2B) and Transcriptional Adaptor 1 (TADA1), discovering new genes involved in CRC that where not characterized until this work was published (38).

Maresch et al., (2016) designed a mouse model to recreate the multiple knock-out genes that generate pancreatic ductal adenocarcinoma. For that, they designed an intra-pancreatic DNA injection and an *in vivo* electroporation method that enables the insertion of multiple sgRNA, a process impossible with viral vectors. They targeted 13 tumor suppressor genes involved in pancreatic cancer, testing different variants and expression levels, monitoring the mice with MRI imaging. They established that the mice that incorporated the sgRNA developed a tumor, in comparison with no tumors in the control group after 24 weeks. They also found that the only targeted gene that wasn't altered was BRCA2, which correlated to the fact that BRCA2 inactivation was needed to inhibit KRAS dependent pancreatic ductal adenocarcinoma, but in absence of TP53, it was able to induce proliferation. Moreover, they used the CRISPR-Cas9 system to screen for metastatic mutations in comparison to other techniques such as Comparative Genomic Hybridization (CGH) arrays (39). CGH arrays are a cytogenetic method for analyzing variations in the number of copies of chromosomes on a genome wide scale without the need for cell cultures. It compares the patient's genome to a reference one, highlighting the differences between them and their exact location (40).

Pancreatical ductal adenocarcinoma was also the spotlight of the study by Bakke et al. (2019), since it is a cancer that, even though many genetic targets have been already been identified, such as KRAS, CDKN2A, TP53 and SMAD4, there is yet a slow progress in the development of effective drugs against this type of tumor, which ultimately translates into poor prognosis. In this scenario, Bakke's team performed a genetic screen using CRISPR-Cas9 to identify novel therapeutic targets. As a result, they were able to find that Proteasome 20S Subunit Alpha 6 (PSMA6) gene was a critical component in cancer cell survival and could respond to Bortezomib (41).

6.2. RECREATING CHROMOSOMAL REARRANGEMENTS

The appeal to mimic the chromosomal rearrangements behind tumorigenesis is explained by the fact that the suppression of the fusion oncogenes that chromosomal rearrangements create seems to be enough to stop cancer growth. In other words, on the contrary of what happens with single mutations (that it is necessary to induce multiple mutations to recreate the cancer environment and, therefore, it is necessary to have multiple targets to stop the cancer from spreading), suppressing one fusion oncogene can be enough to treat the tumor. Once the pathogenesis behind each fusion oncogene is better understood, new drugs used in immunotherapy can be designed to inactivate them (42).

The double-strand breaks formed by the CRISPR-Cas9 technology are able to recreate inter- and intrachromosomal rearrangements. As an example of an **intrachromosomal** rearrangement that CRISPR-Cas9 was successfully able to induce, we can mention the Echinoderm microtubule-associated protein-like 4 (EML4) - Anaplastic lymphoma kinase (ALK) - EML4-ALK, found in lung cancer. The fusion oncogene **EML4-ALK** is the product of an inversion of 2p, and the reproduction of this phenomenon is induced by designing two sgRNA that specifically bind to areas near the EML4-ALK mutation, as represented in Figure 18. The advantage of using CRISPR-Cas9 is that the fusion that occurs are precise, without the incorporation of excessive nucleotides. This is a consequence of the way CRISPR binds to the PAMs, since it doesn't need further end-processing when the cleavage is produced (43).

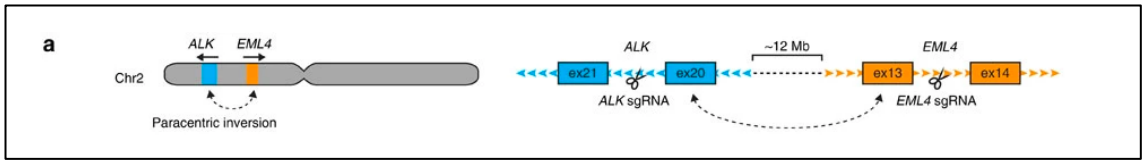

Figure 18. The intrachromosomal rearrangement that forms EML4-ALK, whose pathogenesis is fundamental in lung cancer. (Adapted from Choi and Meyersen, 2014).

On the other hand, the mutations behind Rhabdomyosarcoma (RMS) consist in **interchromosomal** rearrangements of t(2;13) or t(1;13) leading to the subsequent formation of the PAX3/7-FOXO1 fusion gene. This rearrangement can be easily recreated by one sgRNA and CRISPR-CAS9 system. The same sgRNA breaks the PAX3 gene and FOXO1, thus, by non-homologous repair, this two genes are merged (44) Figure 19.

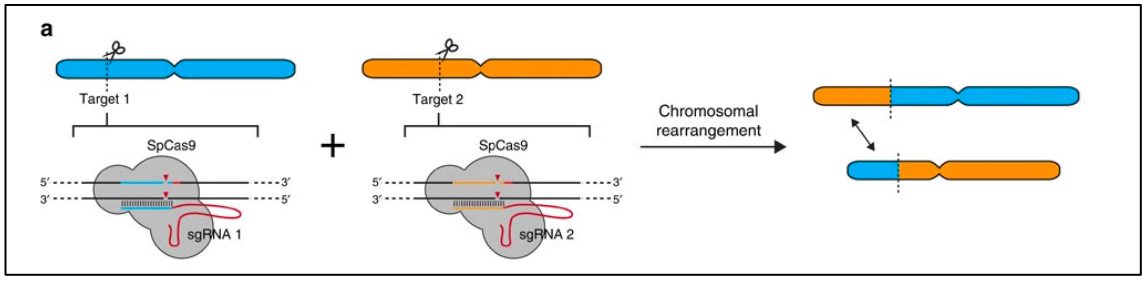

Figure 19. The PAX3/7-FOXO1 fusion gene. (Adapted from Choi and Meyersen, 2014).

In the studied cited before by Maresch et al., 2018, they also recreated various chromosomal rearrangements occurring in pancreatic tumors. More specifically, in this cancer it is known that they are 139 intra-chromosomal deletions and 8 inter-chromosomal translocations per tumor. This study was able to form the following translocations and screen for them with aCGH:

- **Involving chromosomes 4 and 18**
    - Fusion between Cdkn2b and APC: der(4)t(4;18)
    - Fusion between APC and Arid1a: der(18)t(4;18)
- **Loss of Chr17 and Chr19 because of der(19)t(17;19) (39).**

Chromosomal rearrangements have been also characterized in prostate cancer and hepatocellular carcinoma, generating fusion oncogenes Transmembrane Protein 135 - Coiled-Coil Domain-Containing Protein 67 (TMEM135-CCDC67) and Mannosidase Alpha Class 2A Member 1- Feline Encephalitis Virus-Related Kinase (MAN2A1-FER). The translocation behind these fusion oncogenes are 11q14.2-21 and 5q21 (45) (Figure 20 - A). When these oncogenes' breaking points were tackled with the CRISPR-CAS9 system by a pro-drug converting herpes simplex virus type 1 thymidine kinase' (HSV1-tk), the insertion of ganciclovir induced nicks in the previously cited locations, provoking cell apoptosis. Ganciclovir is an analogue of 2'-deoxy-guanosine, which activity depends on the phosphorylation to ganciclovir monophosphate and consequently in ganciclovir diphosphate and triphosphate by the virus kinases in order to inhibit competitively deoxyguanosine triphosphate (dGTP) incorporation into DNA and viral polymerases (46).

The strategy performed in this work as follows: they inserted the HSV1-tk with a recombinant virus that specifically binds to the breakpoint region of those fusion genes. The Cas9 system provokes double-strand breaks that by homologous repair introduce the HSV1-tk in the breakpoint gene, being traduced and transcribed with the TMEM135-CCDC67 gene and forming a HSV1-tk protein. When ganciclovir is internalized in those cells, it is phosphorylated by the virus's kinases into ganciclovir triphosphate, activating HSV1-tk that blocks DNA synthesis. As a consequence, all mouse xenografts studied demonstrated a reduced tumor burden (47) (Figure 20C and Figure 21).

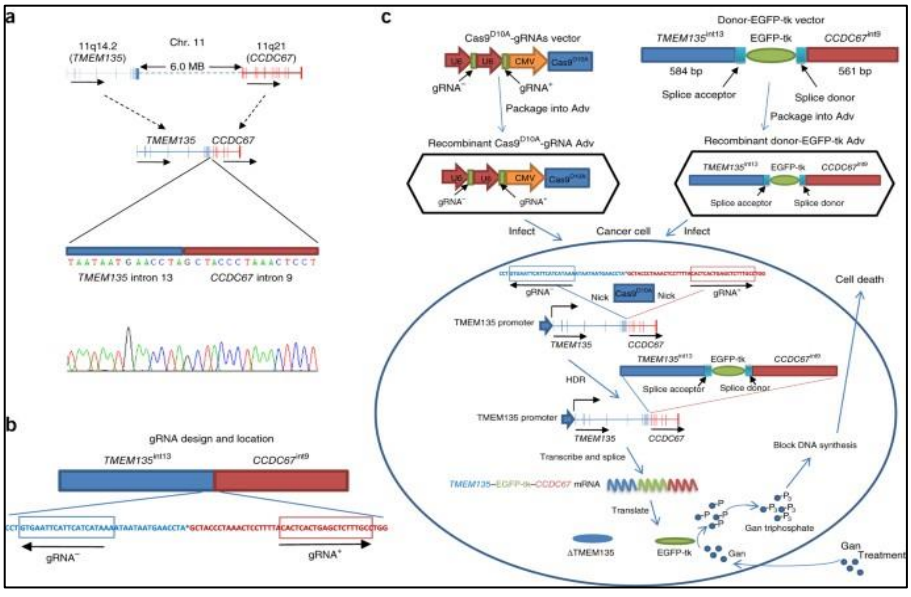

Figure 20. Formation of TMEM135-CCDC67 and introduction in the cell with action of Ganciclovir.
(Adapted from Chen et al., 2017).

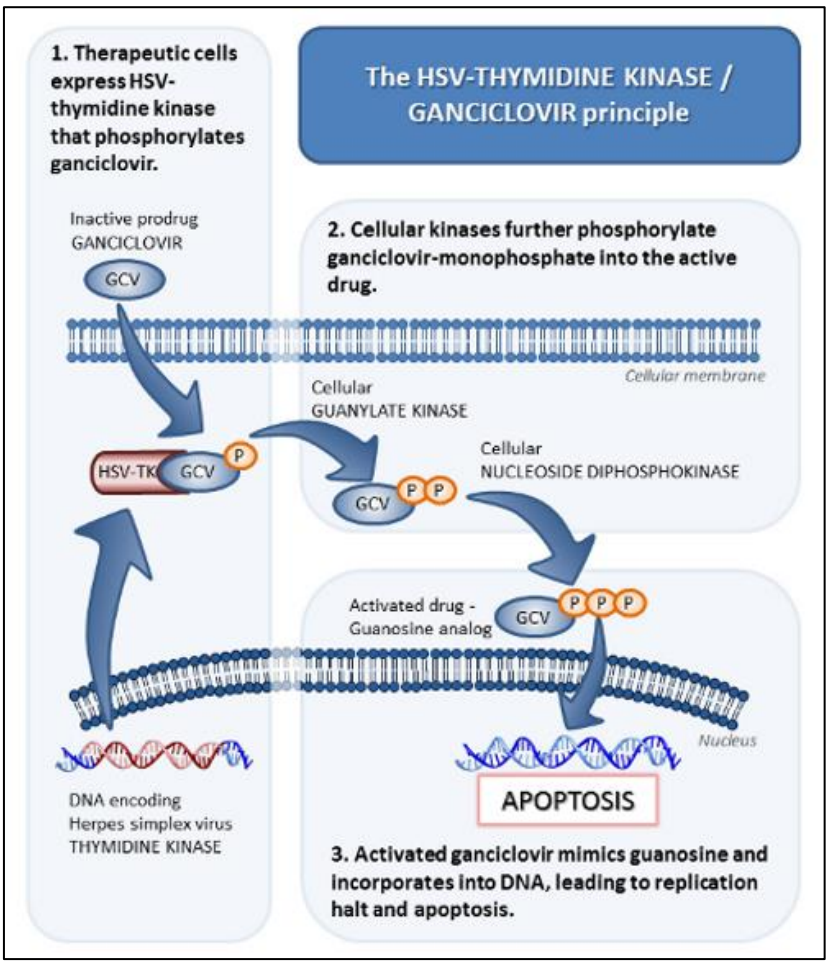

Figure 21. The HSV-Thymidine kinase Ganglicovir principle.

All the chromosomal rearrangements and its correspondent studies are gathered in Table 2.

Table 2. Chromosomal rearrangements in cancer: Studies using CRISPR-Cas9

MUTATION	CHROMOSOMAL REARRANGEMENTS	INTRA O INTER	CANCER	CRISPR-CAS9 DESIGN
EML4-ALK	Inversion 2p	Intra	Lung cancer	Two sgRNA
PAX3/7-FOXO1	t(2;13) or t(1;13)	Inter	Rhabdomyosarcoma	One sgRNA
Cdkn2b - APC:	der(4)t(4;18)	Inter	Pancreatic ductal adenocarcinoma	Multiple sgRNA
APC –Arid1a	der(18)t(4;18)	Inter		
Del Chr17 Chr19	der(19)t(17;19)	inter		
TMEM135-CCDC67	11q14.2-21	Intra	Prostate cancer	One sgRNA
MAN2A1-FER	5q21	Intra	Hepatocellular cancer	One sgRNA

6.3. KNOCK-IN GENES

Mutation screenings can be also developed for knock-in genes or gain-of-function genes. A remarkable study designed by Korkmaz et al., 2015 used the CRISPR-Cas9 technology to screen for **enhancers** that were able to modulate the transcription of p53 and ERα. ERα is an estrogen activated transcription factor whose overexpression is correlated to breast hormone dependent growth and can be treated by using Selective estrogen receptor modulators (SERM) modulators. The enhancers related to these genes once were the non-coding part of DNA that was once classified as "trash", and nowadays is understood to be vital for cell's proper functioning and this study further supports that idea.

Korkmatz et al. were able to characterize the regulation of CDKN1A (formerly known as p21). If the p53 was inactivated, the cell's senescence induced by oncogenes was diminished, such as well the enhancer regions – they were able to demonstrate that the senescence depended on the enhancer, and the enhancer depended on the p53 expression. Moreover, when the enhancer region was broken, there were also a diminished activity of CDKN1A, further emphasizing the importance of the enhancer for the p53 function (48).

7. USE OF CRISPR-CAS9 IN CANCER THERAPY AND IMMUNOTHERAPY

7.1. SUPRESSION OF FUSION ONCOGENES

Above we introduced the importance of being able to suppress fusion oncogenes in order to prevent tumorigenesis. Nonetheless, tackling these mutations is not easy, and the possibility to use the CRISPR-Cas9 mechanism revolutionized this field. In this line, several studies using the CRISPR-Cas9 technology as strategy to attack fusion oncogenes to see if, by employing this technique, they were able to eliminate cancer cells.

Ewing's sarcoma is one of the most frequent malignant cancers in children, and the initial genetic driver behind it is the t(11;22) that forms a fusion oncogene made of the EWS RNA Binding Protein 1 (EWSR1) and Friend leukemia integration 1 transcription factor (FL1) protein – EWSR1-FL1. To inactivate the fusion oncogene, 4 sgRNA were designed to target 2 introns, one in each gene. The cleavage of the introns induced large deletions with frameshift mutations. The design made for this is so specific that it only actuates in cancer cells, so that healthy cells remain unscathed. The results found that the fusion gene inactivation provoked cell's death, reducing tumor burden and mortality. Moreover, when associated with chemotherapy, it created an additive effect that potentiates cell death (42).

7.2. TP53 SUPPRESSION

As previously mentioned, TP53 is an oncosuppressor gene which absence is part of the mutations behind many types of cancer, specially very frequent ones such as lung adenocarcinoma, invasive ductal breast cancer, colon adenocarcinoma and high grade ovarian cancer (49).

There are three different ways in which the TP53 gene can be modified to promote proliferation and growth:

a) **Causing single aminoacidic substitutions and leading to inactivation of the gene**: this is the most common form of mutation of TP53 described until now. It is based in single base mutations that induce a frameshift, producing a nonfunctioning protein. Normally the mutations occur in a "hot spot" – this highlights the fact that it has been selected through time as an advantage for tumor growth (50)(8). "Hot spots" are regions in the genome that exhibit enhanced rates of recombination in comparison to adjacent regions.
b) **Gain of function of TP53**: it upregulates chromatin regulation genes such as methyltransferase 1 (MLL1) and MLL2 and even the group of acetyltransferases as *moz*. The consequence of the upregulation of these transferases is histone modifications. It has been showed the codependence behind this mechanism – TP53 expression is needed for chromatin regulation and vice versa (50).
c) **Epigenetic regulation**: until recently, only "permanent" damage to the DNA has been reported as responsible for tumorigenesis, nonetheless, epigenetic alterations can also have an important role in this process. They consist in chemical modifications of DNA and its folding associated proteins that are able to regulate, up or down, the expression of a certain gene. They are induced by the external cell environment and are less stable when compared to genetic variations, meaning they can be reversible.
   As an example of how vital epigenetics can be for human development, in stem cells, TP53 is normally inactivated since these are highly proliferating cells. Subsequently, when the tissue needs mature cells, the cell's environment induces epigenetic changes to the TP53 gene, stimulating its expression and helping the cell mature to a specific cell linage (51).
   When applied to cancer, normally epigenetic alterations like methylation provoke TP53 gene inactivation. Because they have the characteristic of being reversible, this can be tackled in therapy for inducing tumor suppression. For example, Aurora A Kinase phosphorylates P53 (S212/S312) and methyltransferases produce changes after transcription that invalidates the P53 protein function. When these enzymes are targeted, this process can be overturned – hence the many drugs using this mechanism that are under study (51).

Different strategies have been designed to target TP53. Chira et al., 2018 proposed a method that completely deleted the TP53 mutated gene and replaced it entirely with a functional copy using the homologous recombination. The system is based on a delivery hybrid phage virus induced by doxycycline. Nevertheless, the phage ligand proteins that bind to integrins that are overexpressed in cancer can also be highly present in other cells such as osteoclasts, which represents a problem since it decreases its specificity (52).

Zhan et al. in 2018, in an elegant experiment, created a genetic sensor that traces TP53 reduced expression. First, they identified enhancer regions that were located near all TP53 genes, called p53 bound enhancer regions (P53BER). The P53BER were

configurated to induce expression of sgRNA and Cas9, producing the CRISPR-Cas9 system in the cell. Moreover, a plasmid containing the diphtheria toxin was also introduced in those cells, being able to kill them. Therefore, when the TP53 was expressed, it also expressed the CRISPR-Cas9 system which inactivates the diphtheria toxin by cleavage, saving the cell from toxin death. In other words, when the cells had a TP53 deficiency, they weren't able to inactivate the diphtheria toxin and died. This might provide a future model for therapeutic and detection of TP53 – by reproducing this sensor, cells without TP53 can be killed and prevent proliferation (53).

Despite the potential applications of these tools, using CRIPSR-Cas9 for editing TP53 has encountered some difficulties. In the first place, because of the cell's repair mechanisms that are triggered once a double-strand break is generated, when it is tried to inactivate TP53 with this type of modification, the cell automatically activates apoptosis, loosing edited cells. Furthermore, when TP53 is targeted, is has been shown that is not as specific as it would be desirable – it can also tackle genes like VHL or KRAS, which obligates of a very thorough examination of the edited cells (8).

Recently, Enache et al. studied the effects of Cas9 by itself and remarkably, their experiments showed that its expression in human cells provokes upstream regulation of p53 pathway, with the direct consequence of the expansion of TP53 inactivating mutations as a way to surpass the overexpression. The activation of the TP53 has been demonstrated to be specific, though it is also enhanced by viral vectors and sgRNA. This shed a different light in gene editing with TP53, being more complex than expected and can be a potential obstacle for its usage in clinical trials (54).

7.3. GENERATION OF KNOCK-OUT GENES TO TREAT VIRAL DRIVEN CANCERS

As a contrast from cancers whose origin is not derived from a microorganism pathogenesis, viral driven cancer often has one unique oncogenic mutation. This fact makes this type of cancer as the most suitable for the use of gene editing therapy such as CRISPR Cas9.

A) HUMAN PAPILLOMA VIRUS (HPV) AND CERVICAL CANCER

Cervical cancer is a viral driven tumor whose pathogenesis and correlation to the human papilloma virus (HPV) is very well comprehended and documented, being dependent on the expression of two oncogenes, E6 and E7 (12). These two proteins are produced by high-risk HPV Serotypes. E6 binds to the p53 protein, ubiquitinating, and therefore, targeting it for proteosome degradation thus inhibiting cell apoptosis and promoting senescence. On the other hand, E7 binds to the active retinoblastoma protein (pRb), a family of tumor suppressor proteins, and phosphorylates it, producing its inhibition and the interruption of the E2F pathway, as shown in Figure 22. Both routes are related since the phosphorylation of pRb is also inhibited by p21, a member of the TP53 route (55). The absence of homology with human genome makes these two proteins a very promising target (56).

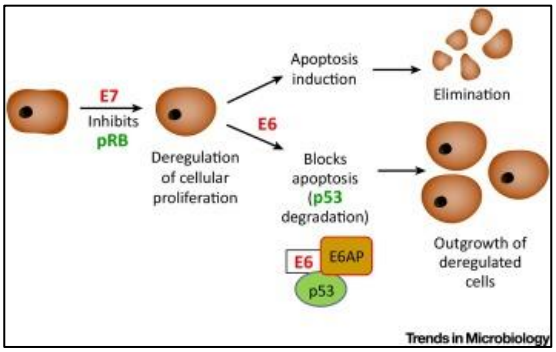

Figure 22. The E7 viral protein inhibits Rb, a oncosuppresor gene. Moreover, E6 blocks apoptosis by promoting p53 degradation. The outcome is an outgrowth of desregulated cells. (Adapted from (57) Hoppe Seyler et al., 2018).

Different studies used the CRISPR-Cas9 technology in an attempt to inactivate this oncoproteins and therefore, to prevent cervical cancer in HPV infected cells. Hu et al., designed a plasmid bearing 4 sgRNAs and expressed them in HPV-16 infected cancer cells so that they induced double-strand breaks in E7. They concluded that the disruption of the E7 gene induced cell apoptosis and restored the tumor suppressor Rb (57). In the same line of research, Kennedy et al., also created 4 sgRNAs that inactivated E6 and E7 in HPV-16 and HPV-18 cancer cell lines. The protein expression was monitored with immunofluorescence and Western Blot methods. The results found were similar to the previous study, underlining a positive outcome in the search for a new target, with promising conclusions *in vitro* (58).

More recently, Zhen et al., took these results as a starting point and went further – not only they tested them *in vitro,* finding accumulation of p53 and p21 and diminished proliferation of cancer cells, but also experimented *in vivo* with cancer mouse models. Specifically, they used female mice with an experimental group that had the E6, E7 genes with the CRISPR-Cas9 gRNA and a control group without the CRISPR-Cas9. They were able to demonstrate weakened tumorigenesis in the CRISPR-Cas9 expressing group (56).

B) HERPES VIRUS FAMILY

The herpes virus family is a complex group of DNA viruses. They are responsible for different types of diseases, variating in range of severity: from simple infections such as cold sores to tumors like Hodgkin's lymphoma. The family can be classified in three groups:

- **Alpha Herpesviridae:** comprehends the herpes simplex virus 1 and 2, responsible for cold sores and genital herpes, respectively. The varicela zoster virus belongs to this group too.
- **Beta Herpesviridae:** cytomegalovirus, the pathogen behind very common congenital effects and chorioretinitis.
- **Gamma Herpesviridae**: Epstein Barr virus (EBV), associated with multiple malignancies such as nasopharyngeal carcinoma, Hodgkin's lymphoma, gastric cancer, Burkitt's lymphoma. This is the herpes virus that has been studied the most using CRISPR-Cas9 for cancer therapy (59).

Even though there are antiviral drugs used to treat these infections such as Acyclovir and its derivatives that tackle the DNA polymerase, they cannot prevent the latent state of the virus since there is not active replication ongoing. The fact that the herpes virus can remain latent in B lymphocytes during years is where the danger lies upon the risk of developing cancer. This is where the CRISRP-Cas9 technology has delivered a glimmer of hope in preventing the oncogenesis (59,60).

Regarding the EBV, the latent state is generated by the formation of an episome with the virus DNA in the infected cell's nucleus. This process is mediated by the union of EBV nuclear antigen 1 (EBNA) to origin of replication zones (60).

Wang et al. (2014) used a cell line with Burkitt's lymphoma and targeted them with sgRNA to produce deletions of the virus DNA. The direct consequence of this was first, the loss of EBV expression, and, therefore, reestablishment of cell apoptosis. The outcome observed didn't guarantee 100% loss of EBV expression, nonetheless, this process can be repeated numerous times and the partial loss could still have a synergic effect in the eradication of the virus because it enables the proper functioning of the human immune system (60).

Van Diemen et al., (2016) followed the same approach by designing two sgRNAs that targeted the EBNA and areas in the EBV origin of replication in latent infected lymphoma cells and used Enhanced Green Fluorescent Protein (eGFP) as a marker of EBV presence. They established a reduction of expression of EBV in the latent cells of almost 95%. The loss of the expression of the EBV in the human cells prevents their cell cycle promoting activity. They were also able to edit the EBV genome using the CRISPR-Cas9 system, by targeting miRNA expressed by the virus (59).

Using a different strategy to arrest EBV expression in latency, Yuen et al. (2015), created a recombinant EBV that didn't express BART (BamHI A rightward transcript) suppressed by two sgRNAs. BART's expression occurs during the latent state of the virus hence, its transcription produces cell proliferation and inhibition of apoptosis, being a hallmark for malignancy. They did this experiment in epithelial cells (61). Finally, again using this technology, they were able to associate the expression of latent membrane protein 1 (LMP1) in nasopharyngeal carcinoma so that its inhibition stopped EBV proliferation (62).

C) HEPATITIS B VIRUS (HBV)

Hepatitis B virus (HBV) is a virus DNA that does its transcription with a reverse transcriptase. It its responsible for a large amount of cirrhosis and, subsequently, is a very important risk factor for hepatocarcinoma.

The drugs available for treatment can be divided into two groups: nucleoside analogous and INF-α. The first ones tackle the synthesis of DNA and the main issue is similar to what happens to EBV: the latent chronic state of HBV is based on a covalently closed circular DNA (cccDNA) that survives on human cells and serves as a template to produce RNA, without needing HBV proliferation and therefore, DNA replication. In other words, the nucleoside analogous have no effect on latent infections and aren't

able to eliminate the HBV from human cells (63). The cccDNA is a viral persistence reservoir and its elimination is essential in the cure of HBV (64).

The HBV replication has 4 open reading frames that produce the Polymerase (P), Core proteins (C), Surface proteins (S), and X proteins (X). The different ways to tackle this genome can be thought out as strategies for eliminating HBV in hepatic cells. The different approaches have been illustrated in Figure 23.

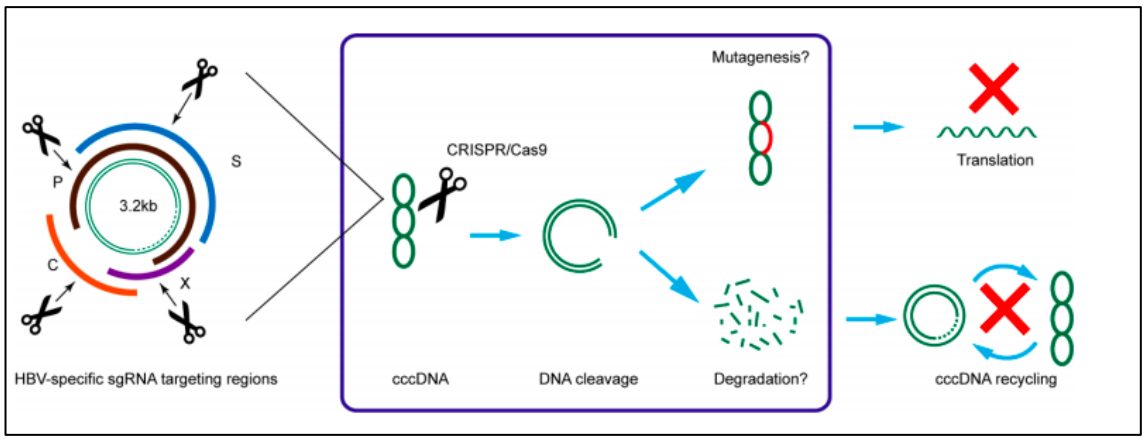

Figure 23. HBV- specific sgRNA targeting regions. (Adapted from Lin et al., 2015).

Lin et al. (2014) generated eight gRNAs against the genotype A to see if they were able to reduce the transcription of Core proteins and Surface proteins: the results found were more promising *in vitro* than *in vivo*, regardless, both approaches were able to induce mutations in HBV (65). Kennedy et al. (2015) directly targeted the reverse transcriptase, surface antigen or core proteins and were able to reduce the accumulation of cccDNA in infected cells by designing 3 sgRNAs in infected hepatocarcinoma cells (66).

In contrast with the studies showed until now whose main target was the genome, Seeger and Sohn (2014) used the HBV receptor, the sodium taurocholate co-transporting polypeptide (NCTP), as a screening method for permissive line cell cultures. Once this cell cultures where determined, they induced double-strand breaks in cccDNA, which provoked NHEJ repair mechanisms, inactivating the HBV genome and reducing the latent state in the liver (67).

In addition to the *in vitro* studies, more recently, Stone et al. (2021), used CRISPR-cas9 technology in mice chronically infected with hepatitis B virus and were able to demonstrate an increased survival in those which HBV genome was edited (68). Moreover, in another study, specific gRNAs were designed to tackle conserved regions of the HBV genome, diminishing the proportion of cccDNA in mice. In contrast of the NHEJ repair mechanism that usually is triggered once the CRISPR-Cas9 system creates the double-strand breaks, when multiple sgRNAs are used, the multiple cleavage sites make it impossible for this kind of repair. Consequently, the cell is obliged to eliminate the DNA templates, and therefore, eradicate the HBV DNA from the infected cells (69)

Using this technology has encountered different obstacles, though. In first hand, some of the impediments are created by the complexity of the infection of HBV:

normally, in chronically infected cells there is more than one cccDNA, besides the fact that is slowly regenerated by replication (68). On the other hand, regarding the use of this technology, the ideal vector still has to be found: it was hypothesized that the most appropriated was an adenovirus vector, but more recent studies implied that the nucleoside analogous could inhibit their transduction (70). Moreover, the possibility of off-target mutations still concerns researchers and has to be token in consideration.

7.4. CELLULAR ENGINEERING FOR CAR-T CELLS

Chimeric antigen receptor T (CAR-T) cells are beginning to revolutionize immunotherapy, especially in hematologic tumors such as acute lymphoblastic leukemia and multiple myeloma. In order to understand how it is possible to apply CRISPR-Cas9 to ameliorate this technology, it is needed to comprehend the structure and functioning of the CAR-T cells: The approach consists of autologous T cells that are engineered *in vitro* and then re-introduced with their new receptors to act against the tumor *in vivo* (71).

More specifically, as shown in Figure 24, the first step in this process is designing the CAR which consists of fragments of the T cell receptor (TCR) bounded to antibody, therefore, the structure is a member of the family of the immunoglobulins. It has an extracellular target-binding protein, a hinge region, a transmembrane protein that anchors the CAR to the membrane and intracellular signals (72). The critical phase in this step is creating the antigen specificity, since this is the basis for the success of the therapy. And, since it is able to directly bind to the antigen, it is not Major histocompatibility complex (MHC) restricted. Next, DNA that encodes to form the CAR is introduced with retroviruses in the T cells. The T cells are cultivated and therefore, selected. Finally, the functioning population of T cells can be infused back to the patient (Figure 24) (73).

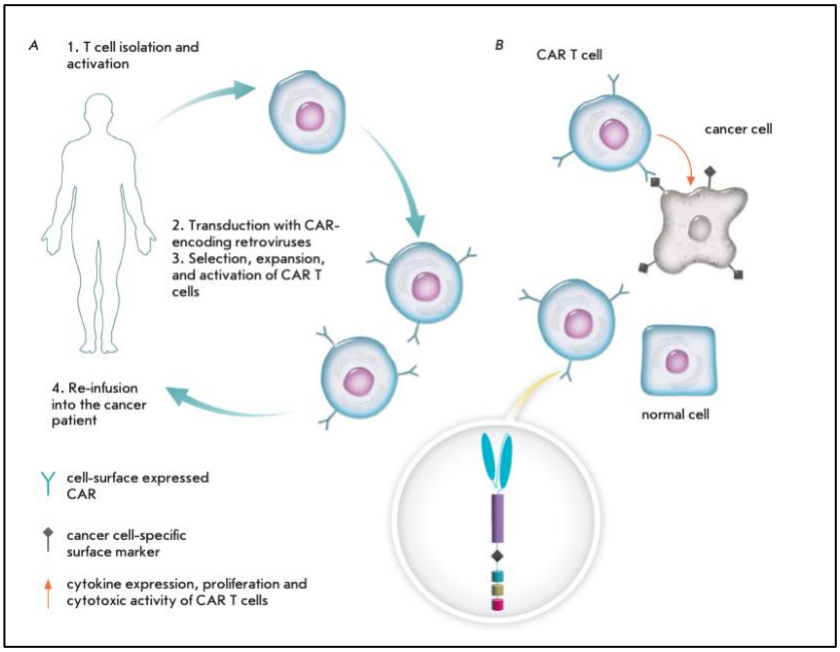

Figure 24. CAR-T cell functioning. The T cells are recognized and isolated, being manipulated in vitro. Then, they are selected and expanded, so they can be re-infunded to the patient. The result is special T cells that can better recognize the tumor and attack it. (Adapted from Kulemzin et al., 2017)

Besides the basic structure of the CAR, many more features can be added to enhance tumor specific cytotoxicity, to mitigate secondary effects, to increase potency, and to overcome tumor immunosuppression. For example, by the inhibition of interleukin genes that mediate cytotoxicity, introduction of chemokine receptors to aid the attachment of the T cell to different sites, and the addition of suicide genes that stablish a security system (72).

In this matter, Jung et al. (2018) knocked out with CRISPR-Cas9 the diacylglycerol kinase (DGK) in order to increase the CD3 signaling in human primary T cells. The DGK is an enzyme that functionally inactivates the diacylglycerol acid, which is responsible for interacting with TCR signaling, therefore, its inhibition has great potential in immunosuppression therapy. They also found that manipulated cells were resistant to suppressor factor such as TGF-β and prostaglandin E *in vitro*. In addition, this technology was also rehearsed in mouse xenographic models with glioblastoma and an enhanced TCR signaling was found (74).

In addition, the tumor micro-environment consists mainly in the production of inhibitory interleukins, such as TGF-β, guaranteeing the immunosuppression and therefore, persistence and proliferation. It has a direct effect in the cytotoxic function of CD8+ cells and favors CD4+ Treg conversion. Tang et al. (2020) used CRISPR-Cas9 to knock-out the TGF-β receptor II (TGFBR2) in CAR-T cells in a double *in vitro* - *in vivo* xenograft model; one being derived by cell lines and the other, in patient derived cells. The results found underly an heightened efficiency of the CAR-T cells even when the tumor was reinoculated (75).

On the other hand, cancer patients not always are the best donors: as a consequence of the disease there can be insufficient number of T cells or they can be non-functioning cells. This highlights the need to be able of creating allogenic universal T cells with minimal graft-versus-host disease (GVHD) or the rejection of the T cells (71), GVHD consisting in an immune response initiated by the recognition of white blood cells from the donors organ by the host's lymphocytes. And the advantage relies not only in the fact that it can be obtained by healthy donors, but that tumor specificity is not needed: they are based on an antibody molecule that recognizes tumor antigens and performs a switch that can bind to the CAR. To improve the generation of universal CAR -T cells, initially many different editing tools were used, and more recently, CRISPR-Cas9 was incorporated to help this process (76).

To avoid the GVHD, it is needed to knockout the TCRα constant (TRAC) and the TCRβ constant, which can be easily accomplished with the CRISPR – Cas 9 and also demonstrated that it can increase T cell potency. In addition, the internalization and re-expression of CAR -T cells as a consequence of multiple exposure of the antigen is more effective, reducing the exhaustion of the activity of these cells (77).

Furthermore, the issue of rejection can also be tackled by eliminating the heavy chains of HLA-1 expressed in human cells, also called β2 microglobulin. And because of the high efficiency of the CRISPR-Cas9 activity, this can be easily done. Besides, to further enhance the reduction of immunogenicity, the TRAC can be also knocked out. Likewise, blocking the PD-1 signaling, which consists of an immunosuppression strategy

developed by tumors, can further ameliorate the design (78). This same idea was improved by using a one-shot CRISPR protocol that could simultaneously knock-out the TRAC, β2 microglobulin and PD-1, speeding the process of engineering the CAR-T cells. This same study also knocked out the Fas receptor (CD45) that has a major role in T cell apoptosis, binding to the Fas-ligand and inducing cell death that downregulates the number of CAR -T cells (79). Figure 25.

On the other side, the absence of HLA-1 makes the CAR -T cells susceptible for natural killer (NK) cells recognition and cytotoxicity. To prevent this from happening, a HLA-E can be express in the cell membrane, functioning as a minimal polymorphic HLA that is sufficient to prevent NK cell activation (80).

Besides the GVHD, the activity of CAR -T cells in the organism can produce cytokine release syndrome, a complication that has a severity range of manifestations, and could be life-threatening. It is a consequence of the binding of the CAR to the antigen, producing the activation of nearby cells such as endothelial cells, producing a massive release of cytokines (81). This side effect can be mitigated by knocking out the granulocyte macrophage colony-stimulating factor (GM-CSF) with CRISPR-Cas9 in these cells, and still preserve the CAR activity (82).

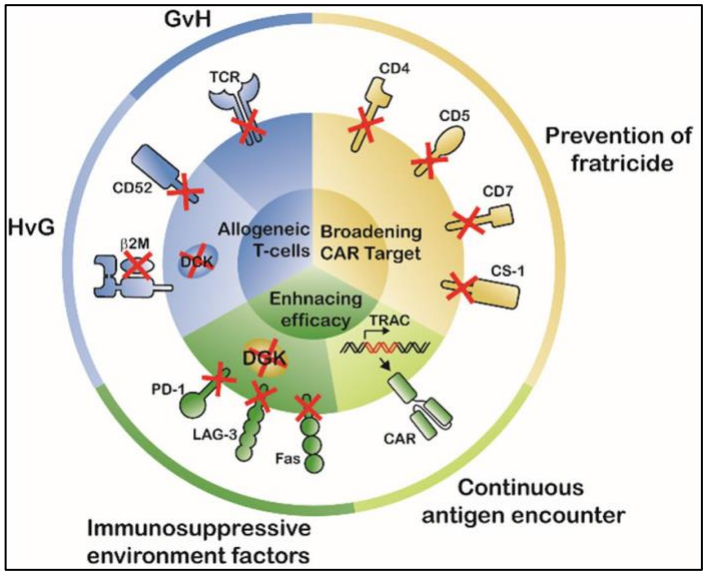

Figure 25. Strategies to better enhance CAR T cells. They can be divided in three different strategies. The first one is the development of allogenic T cells, to save the usage of cancer patients' cells. The main problem is the graft versus host disease that can be prevented by manipulating the TCR. The second strategy is the broadening of the CAR target. Finally, the last strategy is to enhance efficiency, by continuous antigen encounter and immunosuppressive environment factors. (Adapted from Jung and Lee, 2018).

CD7 is a transmembrane glycoprotein physiologically expressed in T cells and NK cells with a role in the co-stimulation derived from the union of the TCR with the antigen, but it is also pathologically expressed in lymphoblastic T-cell leukemia and T-cell lymphomas. Therefore, the CD7 was idealized as a target for immunotherapy against those tumors, which failed as a consequence of the attack on T cells. A way to correct this issue is to design CAR-T cells that have the CD7 gene knocked out with CRISPR-Cas 9, where they were able to expand without compromising the TCR recognition (83).

As promising as this fields is, it also has obstacles regarding its application in a larger scale envisioning clinical trials, such as a large-scale protocol to maximize reproducibility and, therefore, easier techniques are needed. In that matter, besides using the conventional methods for the transduction of CRISPR, like lentiviruses or adenoviruses, a simpler model was developed. It is based on delivering the CRISPR-Cas9 in the form of ribonucleotide proteins (RNP) with electroporation (84,85). And, as previously introduced, the off-target mutations always remain a concern with this technique.

The first in-human trial employing CRISPR-Cas9 technology is being developed, using multiplexing with sgRNA to enhance the efficacy of engineered T cells in cancer, which is explained further below (86).

7.5. IMMUNE CHECKPOINTS

A) PD-1 AND PD-L1

The immune checkpoints have a major role in developing an adequate immune response against cancer cells. They consist of proteins in the T cell's membrane that are able to recognize different ligands in tumor cells, and, therefore, to activate or to inactivate the T cell. The most explored immune checkpoint nowadays is the Programmed Death 1 (PD-1) and Programmed death ligand 1 (PD-L1). The cancer cells express the PD-L1 that binds to the PD-1 in the T cell, initiating an inactivating response and consequently, escaping from the immune system.

Inhibiting the PD-1 – PD-L1 union is a common target for immunotherapy and many tumors use drugs targeting these proteins as a standard treatment. The most well-known cancer in this matter is the non-small cell lung cancer (NSCLC), which can be treated with pembrolizumab, a monoclonal antibody against PD-1. Another way to tackle this union could be by editing out the PD-1 which has been done after series of experiments, culminating in the design of a human clinical trial.

In the first place, Su et al. (2016) demonstrated that ablating the PD-1 with CRISPR-Cas9 using electroporation methods was feasible *in vitro* (87). Furthermore, Rupp et al. (2017) used a similar design, but using a lentivirus form of transduction and also found positive results, being also reproduced *in vivo* in a xenograft model (88). Additionally, this technology has begun to be directly used against cancer cells, for example, in glioblastoma cells (89,90). Likewise, It was also demonstrated to have positive effects when applicated in mouse xenograft models with multiple myeloma (91), hepatocellular cancer (92) and melanoma (93).

Finally, human clinical trials are starting to be developed. Lu et al. (2020) (NCT02793856), knocked-out the PD-1 *ex vivo* and then reintroduced the cells in late-stage NSCLC patients, a process similar to the CAR -T cells previously mentioned. They chose patients that hadn't had an effective response after many lines of treatment, to compensate the risks of off-target mutations. And, moreover, to enhance the safety of the therapy, they performed whole-genome and next-generation sequencing to monitor those mutations. The results support the safety and feasibility of gene editing therapy, with side effects like leukopenia, lymphopenia, fatigue, fever, arthralgia and

skin rash that were well tolerated, and just one case of grade 1 arrhythmia which cause remains unknown was reported. Regarding the concern for off-target mutations, they detected a low rate, being most of them located in introns that didn't produce important alterations (94). Figure 26.

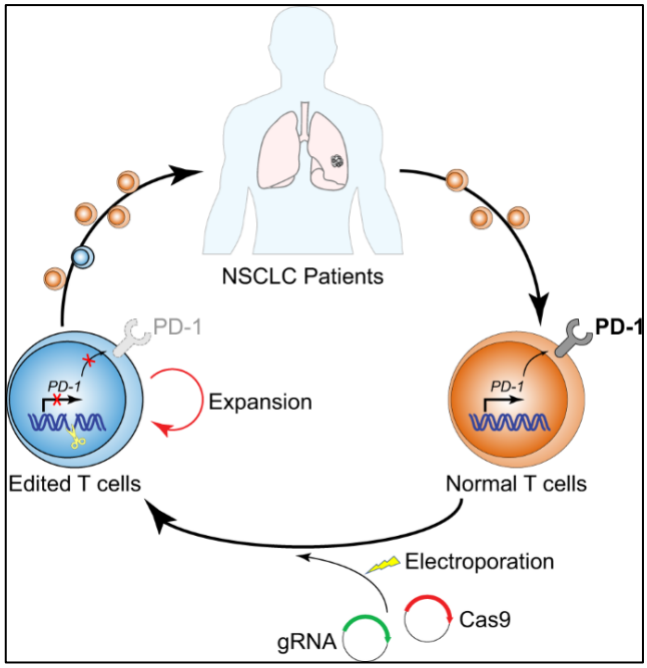

Figure 26. Patients with NSCLC have their T cells removed and the PD-1 edited out. Therefore, they are not able to activate PD-L1 and induce an immunosuppressor response from T cells. (Adapted from Lu et al., 2020)

In addition, Staudtmauer et al. (2020) used the CRISPR-Cas multiplex gene editing to suppress the expression of TCR and PD-1 so as to create a cancer-specific TCR in four patients with advanced cancer. The multiplex editing produced chromosomal translocations, nonetheless, there wasn't important consequences in the patients derived from these mutations (95).

The cycline dependent kinase 5 (Cdk5) serin develops a substantial role in the PD-1 pathway by inducing the production of Interferon-γ (IFN-γ) which induces upregulation of the expression of PD-L1 in cancer cells. As a consequence, the tumor is able to evade the immune system (96). Ardelt et al. (2018) inhibited Cdk5 in hepatocellular carcinoma cells with CRISPR-Cas9 both *in vitro* and *in vivo*, enhancing the efectiveness of Sorafenib and preventing resistance (97).

B) CTLA-4

In a similar perspective previously commented for PD-1 and PD-L1, cytotoxic-T-lymphocyte-associated protein 4 (CTLA-4) also has a pivotal role in immune checkpoints. It is expressed in activated T cells and in regulatory T cells (Treg), and the consequence of binding to B7 (in competition with CD28, which binding leads to a co-stimulatory signal) is an inhibitory signal, preventing the production of IL-2, a proinflammatory interleukin. Therefore, CTLA-4 also represents a very effective mechanism of evasion of the immune response that can be triggered by cancer cells, subsequently, is also a very good candidate to be considered as a major target in therapy. In fact, there is an antibody designed against this protein, called ipilimumab, which its use has been approved for metastatic melanoma (98).

Because of the restrictions of the usage of ipilimumab, a different approach to tackle this target was needed. Hence, as an extension of the CAR-T technology, CTLA-4 can be knocked out with the CRISPR-Cas9, enhancing the anti-tumor activity. This was demonstrated by Shi et al. (2017) *in vitro* and in a xenograft mouse model *in vivo*, which results determined a 40% reduced tumor viability, that could be explained by increased apoptosis and major activity of caspases (91). This same method was replicated in different studies such as the one performed by Peixoto et al. (2016) (99).

C) LAG-3

Inhibiting the two classical immune checkpoints previously described, PD-L1 and CTLA-4, was an important hallmark in immunotherapy, nonetheless, resistance started to emerge very importantly. The way to overcome this issue was to tackle other immune checkpoint previously not confronted – starting a race to better describe these interactions and to develop drugs that could regulate them. In this context, the lymphocyte activation gene-3 (LAG-3) was qualified as promising immune checkpoint to be considered.

Its function is to stablish a co-inhibitory signal that suppresses T cell activation, with a synergic activity to PD-1. In CD4+ cells that are invading a tumor, they are able to inhibit the downstream cascade of activation, leading to an early exhaustion of the attack on cancer cells. As predicted, the double inhibition of PD-1 and LAG-3 can reverse the exhaustion and increases T cell formation (100).

The first drug against LAG-3 was developed in 2006, nowadays there are several treatments under drug trials. Moreover, the combined action of these drugs with nivolumab (an PD-1 inhibitor) showed promising results in melanoma resistant cancer (101). Zhang et al. (2018) used CRISPR-Cas9 to knock-out the LAG-3 in CAR-T cells in cell culture and in a xenograft mouse model, with an efficient approach and a similar functioning to conventional CAR-T cells (102).

8. DRUG RESISTANCE

The main obstacle that cancer treatment has to overcome nowadays is drug resistance. Most of the documented drug resistance is **acquired** and consists of positive selection of the mutated resistant clones, a process analogous to antimicrobial resistance. Until today, the strategy used to diminish resistance is to combine different drugs, however, this can make the results less predictable. Hence, developing new drugs that have different targets can be very expensive and time-consuming, what emphasizes the need of characterizing and understanding the mutations that underly within and to defeat them (103). Because CRISPR-Cas9 can be used to knock-out the genes that allow the cancer cells to escape from responding to drugs and to screen for these mutations, it is becoming a major strategy in order to guarantee cancer remission.

8.1. USING CRISRP-CAS9 IN RESEARCH: identification of potential targets

CRISPR-Cas9 is a simple way to develop a large-scale screening program to investigate gene function, since the specificity of the system depends of short guide sequences, therefore, the employment of oligonucleotide libraries allow to scan a large

pools of genes (104). This can be directly applied to search for potential new targets that can be behind therapy resistance and, consequently, be modified genetically or inhibited by new designed drugs. In this section, all the different applications of this screening method will be explored.

As an example of this application, Wang et al. (2014) designed a loss-of-function genetic screen with a genome-scale lentiviral sgRNA library containing 73,000 sgRNAs to generate knockout collections in two cell lines:

a) *Screening for 6-thioguanine (nucleotide analog) resistance*: most of the screens showed loss-of-function DNA mismatch-repair genes such as MSH2, MSH6, MLH1 and PMS2 with a low frequency of off-targets
b) *Screening for Etoposide (DNA topoisomerase II inhibitor) resistance*: the obvious result was that the loss-of-function of topoisomerase II enabled resistance to Etoposide (since it is its main target). Nonetheless, besides the expected result, they also found that CDK6 is involved in the function of Etoposide, and, therefore, its loss determines resistance. (105)

A similar approach to the use of CRISPR-Cas9 as a genetic screen was developed by Kasap et al. (2014), being it called DrugTargetSeqR. They also screened for two different drugs:

a) *Ispinesib* (inhibits a mitotic motor protein called kinesin spindle protein (KSP), impeding mitosis thus leading to cell cycle arrest): they stablished that acquisition of resistance has no correlation to the DNA mismatch repair genes, in contrast to the results found in resistance for nucleotide analog 6-thioguanine. In a more specific procedure, they demonstrated that mutations of Kinesine-5 were behind the resistance, being either point mutations (such as A133P and D130) or deletions.
b) *YM155* (cytotoxic drug): they weren't able to find any specific mutations correlated to YM155 resistance, leading to the conclusion that the lack of response is due to diminished cell proliferation (106).

Furthermore, Xu et al. (2019) used a genome-wide CRISPR loss-of-function screen to detect the mutations behind the loss of efficiency of gemcitabine in either locally advanced or metastasic gallbladder cancer. As a result, the loss of Elongator complex subunit 5 (ELP5) was identified as a primordial mechanism behind gemcitabine resistance, resulting in an inhibition of apoptosis mediated by p53 because of diminished translation of p53 mRNA. Therefore, they were able to demonstrate that the expression of ELP5 allows to predict the sensitivity to gemcitabine and elucidates it as a possible target for molecular therapy or gene editing (104).

Additionally, the poor response behind osteosarcoma's therapy also incited Wang et al. (2021) to design a genome-wide screen to better identify the molecular mechanisms behind cancer stem cells that compose this tumor. They concluded that Kruppel-like factor 11 (KLF11) acted as a tumor suppressor in those cancer stem cells, and its activation has a synergic effect in chemotherapy. Subsequently and since there are not well stablished markers for chemotherapy success in this tumor yet, the

monitorization of KFL11 can be useful. Their study demonstrated that the CRISPR-Cas9 technology can be efficiently applied for screening cancer stem cells, which until this work was not well explored (107).

High grade serous ovarian cancer is an aggressive tumor with small chances of survival (five-year survival is <50%), in which more than 80% of the overall cases develop chemotherapy resistance, being the treatments mainly based on platinum or taxanes. The process behind this resistance has been studied before and some targets have been identified: mutations in BRCA1/2, overexpression of ATP-binding cassette sub-family B member 1 (ABCB1), also known as Glycoprotein 1 (P-gp) or multidrug resistance protein (MDR-1) and the large family of anti-apoptotic proteins such as B-Cell lymphoma (BCL). Nonetheless, even though these mutations were identified, the direct correlation and pathogenesis behind resistance remained unclear, which motivated Stover et al. (2019) to create a pooled near-genome-scale open reading frame overexpression screen and a pooled genome-scale CRISPR-Cas9 knock-out screen for resistance to cisplatin and paclitaxel. The BCL-2 family were the most clearly proteins associated to resistance, in concrete BCL-XL and MCL1. Therefore, combining drugs against this family of proteins with conventional chemotherapy can be a more efficacious approach to treat this cancer. However, this can be limited by cross-resistance or enhanced expression of other anti-apoptotic proteins (108).

ATR inhibitors are a new approach to cancer therapy, being Berzosertibe the most advanced group representative, a monoclonal antibody being evaluated in a phase I clinical trial showing promising results and safety (109).

Ruiz et al. (2016) developed a wide screen CRISPR-Cas9 to determine the resistance behind the ATR inhibition and their findings proved it was correlated to the deficiency of CDC25 and mutant Cnot8. As CDC25 is considered as an oncogene and it is overly expressed in various tumors, its augmented levels make the cancer cells more susceptible to this drug and could be used in combination with chemotherapy for ameliorating survival and remission. Moreover, they were able to reestablish the sensitivity to ATR inhibitors by using Wee1 inhibitors, a protein that inhibits Cdk1, involved in cyclin-dependent passage of the cell's checkpoints, opening the field for combined drugs with ATR inhibitors and Wee1 inhibitors (110).

Docetaxel and cabazitaxel combined with androgen deprivation therapy is the standard treatment for prostate cancer and specially in castration-resistance prostate cancer. In the last group, the survival results are moderated and seem to be a consequence of taxane resistance (111). Rushworth et al. (2020) conducted an *in vivo* dropout docetaxel sensitation CRISPR screen in prostate cancer that identified 17 genes, being Transcription Elongation Factor A Like 1 (TCEAL1) the most expressed in all the cell lines (112).

All of the resistance mutations cited above are summarized in Table 3.

Table 3. Drug and resistance mutations

DRUG	MO	RESISTANCE MUTATIONS
NUCLEOTIDE ANALOG 6-THIOGUAINE		DNA mismatch repair genes: MSH2, MSH6, MLH1 and PMS2
ETOPOSIDE	Topoisomerase II inhibitor	Topoisomerase II CDK6
ISPINESIB	Kinesin spindle protein inhibitor	Kinesine-5
YM155	Cytotoxic agent	No correlation
GEMCITABINE	Cytotoxic agent	ELP5
CISPLATIN **PACLITAXEL**	Alkylating agent Disruption of microtubules	BLC-2 family
BERZOSERTIBE	ATR inhibitor	CDC25 and Wee1
DOCETAXEL	Disruption of microtubules	TCEAL1
VEMURAFENIB	BRAF inhibitor	NF1, NF2, MED12, CUL3, TADA2B, and TADA1

8.2. APPLICATIONS IN THERAPY

In this section the uses of CRISPR-Cas9 to tackle mutations that produce resistance to different cancer therapies will be discussed, being divided in molecular targeted agents, chemotherapy agents and finally, multi-drug resistance.

A) MOLECULAR TARGETED AGENTS

The emergence of tyrosine kinase inhibitors (TKI) in lung cancer has revolutionized the therapeutical approach offered to patients – besides surgery, radiotherapy and conventional chemotherapy, more direct molecular therapy could be used to improve cancer survival. For lung cancer, the main target is EGFR, which activation induces cell proliferation, neo-angiogenesis, invasion and metastasis and it is inhibited by erlotinib and gefitinib as a first line of treatment. Nevertheless, within 2 years, most patients develop resistance to erlotinib, which eventually was demonstrated to depend on a mutation in position 790 of EGFR, substituting threonine with methionine (T790M). Other common mutations are the deletion of exon 19 and a point mutation in exon 21 that substitutes leucine with arginine at codon 858 (L858R). This provoked the generation of second line and third-line drugs that could bind to that mutation selectively.

Hopelessly, though predictably, resistance has already appeared against the last lines of treatment, which highlights the need to use a different strategy – correct the resistance mutation itself. Tang and Shrager (2016) described this method as “molecular surgery” in which a knock-out of every resistance mutation was created with CRISPR-Cas9 technology (113).

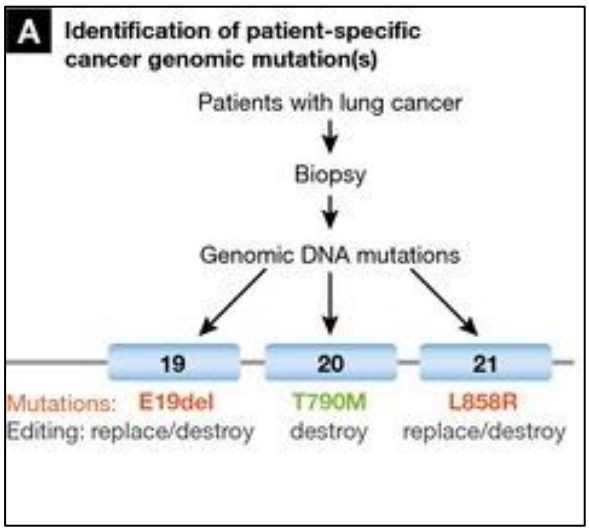

Figure 18. Mutations that confer resistance to TKI. T790M is the mutation responsible for resistante to erlotinib. These mutations can be identified in patients with lung cancer, and they can be replaced or destroyed in order to enhance the sensitivity to TKIs. (Adapted from Tang and Shrager, 2016).

Proteasome inhibitors such as bortezomib are a well-stablished line of treatment for multiple myeloma, nonetheless, again resistance is a main problem mitigating the effectiveness of this therapy. The resistance could be overcome by inhibiting 19S proteasome-associated ubiquitin receptor (Rpn13), a protein involved in the recognition of ubiquitylated proteins and also associated to immune response signaling. This can be achieved by knocking it out with CRISPR-Cas9, and it was demonstrated to have positive results *in vitro* and *in vivo* in multiple myeloma mouse models, defeating proteosome inhibitor resistance (114).

Mantle cell lymphoma is a non-Hodgkin lymphoma that also responds to bortezomib in advanced stages. Chen et al. (2016) elucidated a new mechanism of resistance to this drug, correlated to the Chemokine stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4, expressed in the bone marrow: the resistant cancer cells in a rich environment of ROS upregulate the expression of CXCR4, which stimulates the stem-cell properties that these cancer cells have. Moreover, they also upregulate autophagy for survival in Bortezomib treatment. Therefore, when CXCR4 was inhibited, the sensitivity to Bortezomib was increased (115).

B) CHEMOTHERAPY INTERCALATION AGENTS

Tang et al. (2019) targeted mutated TP53 in osteosarcoma cancer cells with CRISPR-Cas9 and were able to find heightened response to doxorrubicin (116). Moreover, the same result could be achieved when PD-L1 is knocked out with CRISPR-Cas9 (117).

C) MULTI-DRUG RESISTANCE

Multidrug resistance could be a consequence of overexpression of MRD1, an ATP-dependent efflux pump which encodes for P-glycoprotein, a membrane protein that works as a drug transporter. As a consequence, it produces resistance to taxanes, anthracyclines and vinca alkaloids (118). Since its identification, many strategies have been used to minimize its effects:

a) Developing MDR1 inhibitors that could be introduced as adjuvants together with chemotherapy: there are three generations of these drugs with a wide range of response; the first and second failed due to increased toxicity and the third still has unpredictable results in clinical trials (119).
b) Using different pathways for delivering drugs, such as nonviral small interfering RNA (siRNA) and inorganic nanocarriers (120,121).
c) Down-regulation of MDR1: the first strategy to achieve this was the use of siRNAs, with the downside of needing constant downregulation to properly function. Recently, CRISPR-Cas9 offers an alternative, as explored by Ha et al. (2016) and Liu et al. (2016).

Ha et al. (2016) knocked out MDR-1 gene with CRISPR-Cas9 and exposed those cells to doxorubicin, resulting in an enhanced response. However, this could not be potentiated with further injections of CRISPR-CAS9 plasmids, since the MDR-1 is not the only resistance mechanism (118). Liu et al. (2016) applied the same strategy for osteosarcoma: first, they established the relationship between poor survival in this cancer to MDR-1 expression and doxorubicin resistance with a meta-analysis; next, they knocked out this gene with CRISPR-Cas9, restoring its sensitivity to doxorubicin (122). Furthermore, the suppression of CD44 with CRIPSR-cas9 in drug resistant osteosarcoma cell lines culminated in the downregulation of MDR-1 and subsequently, ameliorated chemotherapy response (123).

Additionally, not only MDR-1 overexpression leads to multidrug resistance, since the mutation of numerous resistant determinant genes also have the same result. For instance, when KLF1 is knocked out, it offers resistance to Etoposide and to Gemcitabine. Moreover, when topoisomerase IIA is absent, not only it results in decreased sensitivity to the main target, etoposide, but also to doxorubicin (124).

Likewise, knocking out Urokinase plasminogen activator (uPA) receptor (uPAR) with CRISPR-Cas9 in cancer cells resistant to 5-fluoruracile, cisplatin, docetaxel and doxorubicin showed lessened multidrug resistance (125).

9. HUMAN CLINICAL TRIALS WITH CRISPR-Cas9 TECHNOLOGY

The wide applications of CRISPR-Cas9 in cancer culminated by the increased development of human clinical trials. The first study (NCT02867345) was used in prostate cancer, in a dose-escalation study of *ex-vivo* knocked-out, expanded and selected PD-1 knock-out autologous T cells to achieve the maximal tolerant dose (126). Moreover, another dose-escalation study with PD-1 knock-out T cells was used for metastatic renal cell carcinoma (NCT02867332) (127).

For EBV associated malignancies, the safety of PD-1 knockout EBV- CTL cells is being evaluated in another clinical trial (NCT03044743). It is based on collecting peripheral blood lymphocytes and the PD-1 gene will be knocked out with CRISPR-Cas9 technology, being then reintroduced in the respective patient. The patients will be submitted to 4 cycles of therapy (128).

Another phase 1 safety and efficacy study is being developed on allogenic CRISPR-Cas9 engineered T cells (CTX110) in subjects with relapsed or refractory B-cell malignancies, including Non-Hodgkin lymphoma and B-cell lymphomas (NCT04035434) (129). Finally, a phase 1 trial of autologous T cells engineered to express NY ESO-1 TCR and CRISPR-Cas9 gene edited to eliminate endogenous TCR and PD-1 for multiple myeloma (NCT03399448) (86).

Table 4 summarizes all the human clinical trials developed in cancer using the CRISPR-Cas9 editing method.

Table 4. Clinical trials using CRISPR-Cas9. (Adapted from Mirgayazova et al., 2020)

DISEASE	TRIAL NUMBER	THERAPY	START DATE	STATE
Prostate cancer	NCT02867345	PD-1 knockout T cells	November 2016	Withdrawn
Renal cell carcinoma	NCT02867332	PD-1 knockout T cells	November 2016	Withdrawn
Epstein–Barr virus (EBV) associated malignancies	NCT03044743	PD-1 knockout T cells	April 2017	Active
B cell malignancies	NCT04035434	CTX110 (CRISPR/Cas9-edited T cells)	July 2019	Active
Multiple, melanoma, sarcoma, myxoid/round cell liposarcoma	NCT03399448	NY-ESO-1 redirected autologous T cells with CRISPR/Cas9-edited endogenous TCR and PD-1	September 2018	Terminated

C

10.MARKETING GENE EDITING KITS WITH CRISPR-Cas9

The diversity of applications of CRISPR-Cas9 and, consequently, the high demand of the use of this technology worldwide opened a market of selling **CRISPR kits**. They consist in a variety of different vectors, including plasmids (non-viral vectors), lentivirus, Adeno-Associated virus (AAV) and nanoblades (described by the company itself as “tiny CRISPR ninjas for genome editing difficult cells”-Addgene); synthetic guide RNAs and Cas9 nucleases, customized CRISPR gRNA libraries and mammalian cell line services. Moreover, they fulfill the need for intermediate reagents such as longer DNA oligonucleotides of final reagents of RNA, for example. To summarize, it’s a **ready-to-use** commercial strategy.

All of these tools can be personalized depending on the research it is destined for, with different approaches:

a) **Genome engineering**: including cutting, base editing and performing nicks.
b) **Transcriptional regulation**: activating, interfering and epigenetics.

c) **RNA targeting**: RNA targeting, editing and empty gRNA vectors.
d) **Purify**: to isolate a given genomic locus.
e) **Tag**: to tag a gene of interest.
f) **Visualize**: using fluorescence methods.

Some of the most popular companies that have developed CRISPR-CAS9 commercial kits are GenScript, ThermoFisher Scientific, Addgene, PNA Bio, gBlocks, Sigma and Synthego (130–132).

11. DISCUSSION: CHALLENGES FOR THE FUTURE AND EXPECTATIONS

CRISPR-Cas9 has revolutionized the way we can edit genes. Some years ago, it was an almost impossible task restricted to large laboratories. Nowadays, the technique has been reduced to a simple kit that can be bought off the internet (literally). The main appeal is simplicity and low cost, which can make it accessible and attainable in a large scale. Besides, it can be easily directed to a specific locus and has a high rate of specificity compared to previously used gene editing methods.

For cancer, the applications are almost endless, and they can be grouped accordingly to two main objectives: to better elucidate the mechanisms behind tumorigenesis and therefore, have a better grasp of how cancer works molecularly and to restore those mutations behind this process for therapy.

The ability to easily produce knock-ins, knock-outs and chromosomal rearrangements makes it the perfect tool to recreate the tumor's environment, highlighting the function of well-known genes and discovering the paper of new ones. Likewise, this can be a strategy to find new targets for immunotherapy and to understand the mutations that underly behind drug resistance. Correlating to the last one mentioned, CRISPR-Cas9 can screen for mutations in chemotherapy resisting cell lines and thus, sheds a light into mutations that can be edited or inhibited to maximize tumor regression and survival.

In therapy, CRISPR-Cas9 can be used to knock-out fusion oncogenes that produce common cancers such as Ewing's sarcoma, or can restore the function of TP53, one of the most common tumor suppressor genes inactivated in tumorigenesis. Another very pivotal role of CRISPR-Cas9 is found in viral driven cancers, since the specific inhibition of the virus's proteins can even cure and prevent cancer growth, as was demonstrated for cervical cancer and HPV, Burkitt's lymphoma and VEB, hepatocarcinoma and VHB. One of the highlights and very promising areas for CRISPR-Cas9 is using it to design CAR-T cells, in which the technology is useful in almost every step of the CAR-T generation process: it can be used to create the TCR, to modulate the microenvironment that surrounds the T cells and to prevent GVHD, rejection and cytokine release syndrome after the infusion. In the same line of immunotherapy, the immune checkpoints stablish themselves as a very attractive area for CRISPR-Cas9 gene editing, since it can be used to ablate PD-1 directly without the need of monoclonal antibodies, being this the main subject of an ongoing clinical trial with encouraging results. Similar strategies can be thought out for CTLA4 and LAG-3.

The aim of this technology is to stablish a **personalized medicine**, based on the specific mutations found in the tumor that can be tackled with all the weapons that we desire: chemotherapy, monoclonal antibodies, molecular targeted agents, immunotherapy and **gene editing**. It is a **high precision oncological medicine** that gives high hopes in having better results of progression-free survival and average survival.

The most debated disadvantage of CRISPR-Cas9 and the headlines of discussion of many articles in prestigious scientific journals are **the off-targets effects of CRISPR-Cas9 gene editing**. The controversy started with a paper by Schaefer et al. (2017), now retracted, that exposed the unexpected mutations after CRISPR-Cas9 editing, consisting in a single nucleotide mutation that provoked blindness of the mouse models used in their research (133). In addition, larger scale mutations have also been reported, like in the article by Kosicki et al. (2018) that described large deletions and complex rearrangements in the repair of double-strand breaks induced by CRISPR-Cas9 (134). Rayner et al. (2019) described large-scale deletions and disruptions of targeted locus that was only detectable by using standard Sanger sequencing and locus-specific fluorescence in situ hybridization (FISH) (135). Moreover, the need to screen for these errors has been neglected in the beginning of the usage of this technology. Scientific disputes aside, off-target mutations, in bigger or smaller scale are still an obstacle in using CRISPR-Cas9 further on than mouse models.

The immediate and obvious consequence of the off-target mutations are non-desirable or non-predictable alterations like the described by Schaefer et al. But, in addition, when CRISPR-Cas9 is used to characterize tumorigenesis and to locate new targets for immunotherapy, off-target mutations can suppose misinterpreted results: for instance, there can be a mutation that can be thought out to be pivotal for cancer proliferation, but it was a consequence of the CRISPR gene editing and not produced by the tumor itself.

Many strategies have already been implemented to avoid these inaccuracies in the methodology: using two guide sequences for Cas9 to increase specificity, better designed sgRNAs and screening methods (135). For example, for some locus, CRISPR-Cas9 has low efficiency and has difficult access, therefore, Xi et al. (2015) developed a two-step “pop-in/pop-out” system that allowed them to tag that specific loci, so it can be easily detected by western blots and determined the localization by fluorescence microscopy, avoiding its binding to another location (136). Another important hallmark in reducing off-target mutations is the discovery of **anti-CRISPR (Acr) proteins**, which originally were found in Type I CRISPR-Cas systems, nowadays consisting of over 50 Acr that interact with a variety of cas: Cas3, Cas9, Cas12… As the name suggests, they block the activity of CRISPR-Cas9, using different targets to do so: they can block the interaction or recognition to the PAM site, preventing the binding to the DNA (137,138). They impede the assembly of the crRNA (139) and they also bind to the HNH endonuclease domain to inhibit DNA cleavage (138).They function as a “temporal, spatial or conditional control of CRISPR activity” (140), which can also reduce cytotoxicity of the editing tool in target tissues (141).

The final goal in CRISPR-Cas9 technology is being able to use it widely and with **security in humans**, being the clinical trials the major screening they have to overcome.

The first challenge that can be encountered in human clinical trials is the immugenicity of Cas9 in the host; the Cas9 protein is recognized by the human immune system as an antigen, and it triggers specific T-cells response, besides the innate immune response and humoral immunity (142). Another obstacle is guaranteeing a proper induction of the CRISPR-Cas9 into the host cells, which sometimes can be difficult in certain tissues, requiring non-viral vectors. There are many non-viral methods being used in *in vitro* stages, such as electroporation of nanoparticles, but they do not present the same results when translated to human trials. Many other strategies are being developed such as phage-derived nanoparticles, plasmid encapsulated lipopolymers and liposome-templated hydrogel nanoparticles (103).

As any other editing tool that has emerged in the scientific history, **ethical** concerns always reemerge, especially when considering editing embryos and therefore, inducing mutations that can be passed through generations, and also to avoid ecological impairment and genetic enhancement. Nevertheless, until now, most of the efforts have been directed to somatic cells and monogenic or polygenic diseases, which can postpone this discussion further along.

12.CONCLUSION

CRISPR-Cas9 is a novel gene editing tool characterized by its easiness, efficiency and for being an affordable system. It is based on an ancient immune system that bacteria uses to avoid the infection from viruses, which was modified to achieve its simplest form for programming it to edit human DNA, a discovery that rendered Jennifer Doudna and Emanuelle Charpentier the Chemistry Nobel Prize in 2020. One of the most thought-out applications for its use is in cancer, since it can be effortlessly utilized to manipulate the DNA and recreate tumorigenesis, working towards a better understanding of it and, therefore, elucidating new targets for therapy. Likewise, it can directly correct mutated genes that are responsible for carcinogenic events or stablish new mutations that could revert them. The vast variety of applications culminate in assaying them in human clinical trials, which consists in the last final step in expanding its use and stablishing it as a high precision oncological medicine with major improvements in regression and survival.

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