

---

# FULL CHAPTER REVIEW REPORT

---

CHAPTER: CRISPR-ERA for switching off (onco)genes  
AUTHORS: Manuel Sanchez-Martin and Ignacio Garcia-Tuñon  
ACADEMIC EDITOR: Aditi Singh  
DATE: 11.07.2018, London

## TITLE

Chapter title:

Needs revision

Suggestion:

The current title “CRISPR-ERA for switching off (onco)genes”, accurately reflects the content of the paper, and the level is appropriate to our readership.

However, Please double check the titles. Was the title Given during proposal submission the same? It appears to me that it was initially “Highly efficient CRISPR method for generating nulls zygotes and cell lines.”. Please correct the proposal title.

## ORIGINALITY

Is the chapter acceptable in its current form?



Yes

## **METHODS USED**

Are the research methods and analysis well explained?

Yes

## **KEY RESULTS**

Is the discussion carried out in a satisfactory manner?

Yes

Does the conclusion support the presented research ?

Yes

## **REFERENCES**

Is the reference list adequate?

Yes

## **EDITOR'S COMMENTS**

In this review titled “ CRISPR-ERA for switching off (onco)genes”, the author discusses the utilization of CRISPR technology for gene silencing and their application in gene therapy, particularly focusing on oncogenes.

They begin with a wide overview of gene suppression therapies involved in treating cancer, followed by a generic overview of the potential of CRISPR-Cas9 technologies in gene editing. The author then tries to marry the two ideas and give specific examples how in the past researchers have successfully utilized the potential of CRISPR mediated gene editing for therapeutic applications (specifically targeting oncogenes).



The idea is well supported by the references and figures, and the authors have successfully reasoned that the use of a sgRNA-targeting splicing site could improve the null result for in vivo gene therapies. This strategy could be adopted to abrogate in vivo the oncogenic activity involved in tumor maintenance.

The chapter is interesting and insightful, and very well written.

Specific comments:

- The current title “CRISPR-ERA for switching off (onco)genes”, accurately reflects the content of the paper, and the level is appropriate to our readership.

However, Please double check the titles. Was the title Given during proposal submission the same? It appears to me that it was initially “Highly efficient CRISPR method for generating nulls zygotes and cell lines.”. Please correct the proposal title.

- The abstract also presents an accurate synopsis of the paper.

- The introduction section is scientifically well-written in an engaging and lively style.

I recommend the manuscript to be suitable for publication in its current form.

## EDITOR DECISION

ACCEPT



## **CRISPR-ERA for switching (onco)genes off.**

Ignacio García-Tuñón<sup>1,2</sup> and Manuel Sanchez-Martin<sup>2,3,4</sup>.

1. Centre for Cancer Research (IBMCC), Salamanca, Spain.
2. Biomedical Research Institute of Salamanca (IBSAL), Spain.
3. Transgenic Facility of Nucleus, University of Salamanca, Spain.
4. Department of Medicine, University of Salamanca, Spain.

### **Abstract**

Genome editing nucleases like the popular CRISPR/Cas9 allow generate knock-out cell lines and nulls zygotes by inducing site-specific DSB within a genome. In most cases, when a DNA template is not present, the DSB is repaired by non-homologous end joining (NHEJ) resulting in small nucleotide insertions or deletions that can be used to construct knockout alleles. However, for several reasons, these mutations do not produce the desired null result in all cases, generating a similar protein with functional activity. That undesirable effect could limit the therapeutic efficiency of gene therapy strategies focused on abrogating oncogene expression by CRISPR/Cas9 and should be taken in account. This chapter reviews the irruption of CRISPR technology for gene silencing and its application in gene therapy.

**Keywords:** Gene therapy, knock-out, null allele, oncogene silencing, CRISPR technology, gene suppression.

### **1. Introduction**

#### **GENE SUPPRESSION THERAPIES IN CANCER: AN OVERVIEW**

Gene therapy, which was initially developed for the treatment of genetic (primarily monogenic) diseases, has been mainly focused on cancer therapy. In result of this, meanwhile more than 65% of all gene therapy trials worldwide (Figure 1) are aiming at the treatment of solid and also hematological malignancies[1]. As a consequence, cancer gene therapy represents the predominant field of basic research as well as clinical activities (Table 1)[2].

Different strategies at different molecular levels (figure 2) have been employed to treat malignant diseases during the last decades such as specific drugs inhibitors at protein level, gene suppression therapies at mRNA level or genome editing nucleases at DNA level[3].

The ability of several drugs to inhibit the activity of a targeted oncoprotein has been exploited as a therapeutic approach for a variety of malignancies, the best example is imatinib mesylate, a tyrosin-kinase inhibitor (TKI) indicated for the first-line treatment of Chronic Myeloid Leukemia (CML). The advent of imatinib mesylate from the end of the 20th century onward has revolutionized CML prognosis reaching an overall survival (OS) rate of 88% after 5 years, whereas previous nonspecific treatment produced OS rate of 57%[4]. Unfortunately, despite increased efficacy and better clinical responses, many patients receiving targeted drugs exhibit a poor initial response, develop resistance or undergo relapse after initial success. Besides, except a subgroup of patients who

achieve a deep and sustained molecular response, TKI therapies would need to be continued indefinitely because TKIs do not completely eliminate the leukemia stem cells (LSC) and they persist even during effective TKI treatment [5].

Alternative onco-protein inhibition approach arises from the ability of some small RNAs to fold into three-dimensional structures which can then bind to proteins and thereby inhibit them in a manner similar to protein antagonists[6]. This is the logic behind the use of RNA 'decoys' or RNA aptamers. Recent preclinical and clinical data support the potential activity of a 45-nucleotide-long RNA aptamer (NOX-A12) that specifically antagonizes the CXC chemokine ligand 12/stromal cell derived factor-1 (CXCL 12/SDF-1) which is a key regulatory chemokine for the migration of leukemic stem cells into the bone marrow [6]. This inhibition of binding of SDF-1 to its receptors can lead the leukemic stem cells to re-enter the cell cycle and become vulnerable to chemotherapeutic attack.

Additional gene suppression therapies are focusing on the intervention of gene transcription and translation, which represent vital elements for cancer growth, spread, survival and therapy resistance. Ribozymes, antisense oligodeoxynucleotides (AS-ODNs) and short interfering RNAs (siRNAs) are an emerging class of targeted DNA-based pharmaceuticals. Ribozymes, a subset of catalytic RNAs, can be artificially synthesized and used to specifically suppress gene function. They can also be used to validate disease-related genes as potential targets for new therapeutic interventions. Their ability to cleave mRNA to prevent protein synthesis enables them to find applications in cancer and virology. Transcripts of genes of different function have been targeted by AS-ODN gene therapies such as c-myc, c-raf, c-fos, H-ras, Her2/neu, bcl-2, VEGF and Ang-1. The use of AS-ODNs was shown to successfully inhibit gene expression in association with tumor growth inhibition, radiosensitization or chemosensitization [7-9]. The use of siRNA technology provides is another novel approach for targeted sequence-specific suppression of target gene expression. In this system, siRNA stability and proper delivery are key factors for successful application. In-vitro and in-vivo studies with siRNA targeting PKN3 mRNA have shown success inhibiting tumor progression and metastasis in lung and mammary carcinoma models[10]. Nonetheless, inefficient/complete silencing and transient effect represent the major challenges for cancer gene therapy with gene therapies mediated by Ribozymes, AS-ODNs or siRNAs[2]. Other key challenges that need to be addressed for the successful translation of these approaches will be their delivery to the site of action, choice between direct delivery and the use of a vehicle, mass production at low cost, more clearly defined pharmacokinetics, ability to produce sustained long-term effects, immunogenicity and toxicity (including inappropriate or excessive expression).

With the recent explosion of genome editing tools, including clustered regularly interspaced short palindromic repeats and its nuclease associated protein Cas9 (CRISPR/Cas9), the landscape on suppression techniques has dramatically changed. Although CRISPR/Cas9 is similar in action and efficacy to protein-based targeted nucleases, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs)[11], the ease of design and testing of these

reagents through the construction of single-guide RNAs (sgRNAs) has made gene editing available for a wider variety of users and applications.

CRISPR/Cas9 performs its work at DNA level and it has the advantage of providing permanent and full gene knockout while AS-ODNs and siRNAs only silence genes transiently because they are working at mRNA level [12, 13]. CRISPR/Cas9 cuts DNA in a sequence-specific manner with the possibility of interrupt coding sequences and has thereby made possible turn-off cancer drivers that were not previously feasible in humans [14, 15]. This notable application of permanent gene disruption is due to cellular mechanisms involved in double-stranded break (DSB) repair.

## 2. CRISPR-Cas9 TECHNOLOGY, DSBs AND GENE INTERRUPTION

CRISPR/Cas9 system allows sequence-specific gene editing in many organisms and nowadays is the best tool to generate cell lines and animal models of human diseases. The main advantages of this technology are its simplicity, versatility and efficiency compared to other gene-modifying technologies. CRISPR/Cas9 technology is usually used for introducing targeted double-stranded breaks (DSBs) in any biological system[16] and the only requirement for Cas9-mediated DNA recognition and cleavage is the presence of a short protospacer adjacent motif (PAM) immediately 3' of the targeted DNA sequence[17].

Following the creation of a double-stranded break (DSB) within the coding sequence of a gene, mechanisms of DNA repair can induce insertions and deletions (indels) resulting in frameshift or nonsense mutations[18]. Basically, the repair of DSBs involves four possible mechanisms (Figure 4). The first mechanism is a classical nonhomologous end-joining (C-NHEJ). In this mechanism, the DSB is repaired by blunt end ligation independently of sequence homology and requires DNA ligase IV action (Figure 1A). C-NHEJ can occur throughout the cell cycle but is dominant in G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub> and it is associated to 1-4 bp deletions [19] which could produce frameshift mutations. Alternatively, the DSB end can be resected, leaving 3' single-stranded DNA (ssDNA) overhangs. The resected DSB can be repaired by three possible mechanisms: homologous recombination (HR), single-strand annealing (SSA), and alternative end joining (alt-EJ). HR predominates in the mid-S and mid-G<sub>2</sub> cell cycle phases, where the amount of DNA replication is highest and when the sister template is available[20]. Because HR uses a template for repair, it requires strand invasion mediated by the recombinase RAD51 (Figure 4)[21]. Taking in advantage this property could be possible to edit mutations delivering the appropriate template joined to CRISPR/Cas9 system inside the mutated cell. The resected DSB can also be repaired by mutagenic repair pathways, namely SSA or alt-EJ. SSA mediates end joining between interspersed nucleotide repeats in the genome and involves reannealing of Replication Protein A (RPA)-covered ssDNA by the RAD52 protein. SSA is typically associated with large deletions (Figure 4)[21]. Alt-EJ mechanism remains unclear and it has apparent predilection for joining DSBs on different chromosomes generating chromosomal translocations and mutagenic rearrangements (Figure 4)[22]. Early evidence for alt-EJ came from

studies showing that yeast and mammalian cells deficient in C-NHEJ were still able to repair DSBs via end joining[23].

As consequence of its efficiency inducing DSB, CRISPR/Cas9 technology has been launched as the “gold standard” for creating null alleles in both, “in vivo” and “in vitro”, situations. These null alleles can arise from frameshift mutations, premature stop codons or/and non-sense mediated decay on the target gene, which result in loss-of-function. Currently, CRISPR/Cas9 is extensively used to engineer gene knockouts, but due to the variable size of NHEJ-induced indel, generating a full KO in one step is not always achieved with high frequency. In fact, full KO generation requires off-frame mutations in both alleles and this is a likelihood issue because several mutations could preserve the reading frame (e.g. +3 or -3 mutations). In those assays, where the knockout cell can be selected, or the null allele of the animal model can be segregated, this undesirable effect can be irrelevant[24]. The first clinical trial using CRISPR for gene suppression and cancer therapy has enrolled the first patient at Sichuan University’s West China Hospital in Chengdu in 2016[25]. In this study, the safety of PD-1 knockout CRISPR-engineered T cells ex vivo is evaluated in treating metastatic non-small cell lung cancer that has progressed after all standard treatments. Patients enrolled in the gene-editing trial are providing peripheral blood lymphocytes and PD-1 knockout of T-cells by CRISPR/Cas9 is performed ex vivo. In this trial, the edited lymphocytes are selected, expanded and subsequently infused back into the patients (Figure 5).

Nevertheless, there are several situations, either “in vivo” or “in vitro”, where cell-selection and expansion is not an option and to obtain a high knockout/gene inactivation efficiency is crucial[26, 27]. Hematological cancer therapies based on specific oncogenic silencing within primitive pluripotent stem cells could be the best example of these situations. In that pathological cell-context, high efficiencies of interrupting an oncogenic open reading frame (ORF) could be an effective therapeutic option. It would even be more important for those tumors directed by a single oncogenic event, as several leukemia or sarcomas, which are directed by specific fusion oncoproteins[28] [29].

#### CRISPR-Cas9 TECHNOLOGY FOR DISRUPTING FUSION ONCOGENES

Fusion onco-proteins resulting from chromosomal rearrangements are known to drive the pathogenesis of a variety of hematological neoplasms such as CML. CML resulted from a reciprocal translocation between chromosome 9 and 22[30, 31]. This translocation fuses the *ABL1* gene on chromosome 9 to the *BCR* gene on chromosome 22, resulting in *BCR/ABL* fusion gene. The *BCR/ABL* fusion gene product is a cytoplasmic 210-KDa protein with upregulated tyrosine kinase activity that is considered essential for growth and survival of the leukemic cells[32]. As we previously mentioned, the discovery of *BCR-ABL*-mediated pathogenesis of CML provided the insight for the design of an inhibitory agent that targets BCR/ABL kinase activity such as imatinib mesylate. However, a substantial proportion of CML patients may not achieve the desired response

or may eventually lose an adequate response to these drugs[4]. ). A recent study focused on BCR/ABL fusion shows CML is an ideal target for CRISPR-Cas9-mediated gene therapy. The study shows a CRISPR-Cas9 application for truncating the specific BCR-ABL fusion (p210) in an “in vitro” cellular model [15](Figure 6).

In this study, a non-tumorigenic cell line (BaF3), which needs IL-3 to survive and proliferate[33], is transformed with the fusion oncogene BCR/ABLp210 (BaF3-p210). The human BCR/ABL oncogenic fusion confers BaF3 the ability to survive and proliferate in the absence of IL-3 and forms tumors in a xenograft model. Three custom-designed sgRNAs were used to genetically inactivate the BCR/ ABL oncogene. These specific sgRNAs directed Cas9 to the BCR/ABL fusion sequence (Bcr-Abl sgRNA) or to the Abelson tyrosine kinase sequence (Tk-Abl 1 sgRNA and Tk-Abl 2 sgRNA) (Figure 7). Lentiviral infection assays were performed with each CRISPR/Cas9 reagents to generate three different BaF3-p210 cell lines with the potentially edited BCR/ABL oncogene at the expected cleavage expected point each one.

The CRISPR-Cas9 system efficiently induced different mutations at the expected cleavage expected point, giving rise to a three different BaF3-p210 cell lines (CRISPR-BaF3-p210) with several altered BCR/ ABL sequences.

As a result, a significant increase in cell death was observed in all CRISPR-BaF3-p210 cell lines in the absence of IL- 3, compared with BaF3 parental cells or mock BaF3-p210 cells (Figure 8).

Xenografts experiments were carried out to determine whether the tumorigenic capacity of was also blocked by the action of CRISPR/Cas9 system. Mice injected with the three CRISPR-BaF3-p210 cell lines gave rise to significantly smaller subcutaneous tumors than those produced by the non-edited cells (Figure 9).

As expected, these small tumors were composed of non-edited cells, edited cells with +3/-3 bp indels (or multiples), or cells with non-frameshifts mutations. This result indicated that a specific cellular selection or more specific sgRNAs should be necessary before a potential gene therapy in human. For this purpose, a CRISPR-BaF3-p210 cell line derived from a single cell (CRISPR-BaF3-p210-SC) carrying an 8-bp deletion (Figure 9) was selected to test the tumorigenic capacity. No tumor growth was observed in any mice injected with cells derived from the single-edited cell line (Figure 9).

#### CRISPR/CAS9 AND KNOCKING-OUT GENES IN MOUSE

An option to improve the knock-out effectiveness could be using two or more RNA guides at the same time to knockout the oncogene allele at different key sites trying to guarantee the null result. This approach is commonly used for knocking-out genes in animal models such as mice. Besides, using two sgRNA guides allows distinguish the mutant pups by a simple PCR. An example of this is the generation and genetic characterization of Six6os1-deficient mice[34] (Figure 10).



Unfortunately, the possibility of using several RNA guides at the same time is quite limited in gene therapy especially when adeno-associated virus vectors are being used. The main issue is due to construct size limitations and for this reason other Cas9 orthologues are being used to introduce the nuclease coding sequence, one promoter and a single RNA guide[26, 35].

## CRISPR/Cas9 DELIVERY AND GENE THERAPY

The CRISPR/Cas9 complex can be introduced into the cell in the forms of DNA, RNA, or protein into Cultured Cells and Single-Cell Embryos[36]. The DNA encoding Cas9 and gRNA can be delivered into the cell using the plasmid and viral expression vectors. RNA or protein has been introduced through microinjection, liposome-mediated transfection, electroporation, or nucleofection. However, the delivery formats of mRNA and protein pose certain technical challenges *in vivo* and the viral-based *in vivo* genome editing remains a popular choice to achieve stable or elevated expression of Cas9 and its sgRNA[37]

Given the great potential of viral vectors in gene and cell therapy, five major classes of viral vectors—retroviruses[38], lentiviruses[39, 40], adenoviruses[41, 42], AAVs[43, 44], and baculoviruses[45, 46]—have been employed to deliver CRISPR components into mammalian cells for targeted genome editing. The advantages and disadvantages of using these viral vectors for *in vivo* delivery of the CRISPR transgenes have been extensively reviewed[43, 47-49]. In Table 2 we listed general characteristics and applications of various viral delivery vectors).

Currently, adenoviral vectors and  $\gamma$ -retroviruses are the most used delivery system in gene therapy (Figure 11; Table 3) [1]. For Cas9 delivery, adenovirus (ad) and retro/lentivirus (rt/lt) based vectors have the advantage of packaging sizes of up to 30 (ad) and 7 kilobases (rt/lt), allowing to accommodate the SpCas9 gene (~4.2 kb), one or more sgRNAs and the cis-acting regulatory sequences required for efficient expression. Nevertheless, several disadvantages such as low titers (rt/lt), the insertional oncogenesis (rt/lt), generation of a replication-competent lentivirus (rt/lt), immunogenicity and toxicity (ad), are risks that should be taken account for “*in vivo*” gene therapy. In contrast, the AAV system provides major advantages for research and therapeutics, including a very low immune response and toxicity. Besides, AAVs remains into the cell as episoma avoiding insertional mutagenesis by random integration into the host genome. Indeed, there are not human diseases related to them and they can exist long-term as concatemers in non-dividing cells for stable transgene expressions[50]. Given this, AAV is thought to be one of the most suitable viral vectors for gene therapeutic applications and gene transfer *in vivo*. However, two limitations restrict its use: packing size and tropism. AAV has a packaging capacity of only ~4.8 kb. This makes it impossible to express the ~4.2-kb SpCas9 gene and the sgRNA, from a single AAV vector. One approach is to use two AAV vectors: one to express SpCas9 and the other encodes one or more

sgRNAs[44]. A second approach is to use a different, smaller Cas9, for example, the ~3.2-kb Cas9 gene encoded by *Staphylococcus aureus* (SaCas9)[35, 51]. In this sense, single AAV vectors able to express SaCas9 and one sgRNA have been described and appear potentially very useful for *in vivo* gene editing. A single AAV vector with U6-driven sgRNA and a TBG driven SaCas9 expression cassette was used to target the cholesterol regulatory gene *Pcsk9* in the mouse liver. In this study the authors observed >40% gene modification, accompanied by significant reductions in serum *Pcsk9* and total cholesterol levels[35].

A second concern with AAV vectors is their limited tissue tropism, although this has gradually expanded with the identification of additional AAV variants from different species and the derivation of AAV recombinants with enhanced tropism for specific tissues[52, 53]. AAV serotypes with a strong tropism for hepatocytes, neurons, and epithelial and endothelial cells have been described, but AAV variants able to efficiently infect HSC or lymphoid cells are yet on-going [54].

All these advantages have increased the number of the studies used AAV vectors to deliver the CRISPR components into animals and clinical trials for gene therapy.

#### CRISPR-Cas9 sgRNAs: “*SUPERGUIDES*” FOR INTERFERING (ONCO)GENE EXPRESSION

When a cancer cell is the target, a delivery strategy that can result in the expression of Cas9 and an oncogenic-specific sgRNA in all infected cells would be desirable. That is especially critical for “*in vitro*” gene therapy where the expansion processes from a selected edited cell is not available. Similarly, it is also crucial for *in-vivo* approaches in cancer therapies focused on disrupting a driver oncogene. If the efficiency of CRISPR/Cas9 reagents delivery to the cancer cell is acceptable, the key step for success lies in the effectiveness of a specific sgRNA for knocking-out the oncogene. In that way, a vast majority of knockout studies where the edited cells or mice can be selected, the sgRNA are targeting different positions within the elected exon avoiding boundaries. Most cases of them the designs are following off-targets criteria. However, for all those cases where cellular selection is not an option and only one sgRNA can be used, the null effect could be increased with sgRNA targeting exon boundaries. It seems reasonable to think that following this strategy, together with the probability of producing frameshift mutations, it should be added the probability of breaking the canonical pre-mRNA splicing (Figure 12).

It has long been known that mutations in splice site consensus sequences can affect pre-mRNA splicing patterns and can lead to generate null or deficient alleles[55]. In fact, pioneered genetic studies indicated that many of the thalassemia mutations in the  $\beta$ -globin gene affect splice sites and give rise to aberrant splicing patterns[56, 57]. Recent studies have demonstrated that a splicing mutation in STAR gene produces an aberrant protein and this splicing mutation is a loss-of-function mutation[58]. Besides, nonsense-mediated mRNA decay (NMD), a conserved biological mechanism that degrades transcripts containing premature translation termination codons, could help to achieve the null effect when a DSB is induced in splice-sites. In addition to transcripts derived from

nonsense alleles, the substrates of the NMD pathway also include pre-mRNAs that enter the cytoplasm with introns intact[59]. Recently have been identified several mutations of the splice donor sites resulting in a gene loss-of-function. A novel mutation on a splice donor site and predicted to lead to skipping of exon 10 of PLA2G6 gene was found in a homozygous state in infantile neuroaxonal dystrophy patients. This variant was correlated with a very strong loss-of-function supporting evidences for its pathogenicity[60]. Mutations in the ectodysplasin A1 gene (EDA-A1) on the splice donor site have been described in patients with hypohidrotic ectodermal dysplasia. The mutation resulted in the production of a truncated EDA-A1 protein caused by complete omission of exon 3. This novel functional skipping-splicing EDA mutation was considered to be the cause of the pathological phenotype[61]. Studies in a family with premature ovarian failure identified a variant that alters a splice donor site. This variant resulted in a predicted loss of functional MCM9 gene, a gene implicated in homologous recombination and repair of double-stranded DNA breaks[62].

As we have mentioned before, not all indels targeting the exon coding sequences necessarily lead to premature stop codons. However, if DSBs are induced near boundaries on the target exon canonical splicing pathway could also be altered. In that case, linked to the probability of producing frameshift mutations, it should be added the probability of interfering the canonical pre-mRNA splicing (Figure 12 B). If the result of the CRISPR/Cas9-induced mutation did not produce a frameshift mutation, at least this strategy would add the probability of producing non-functional oncogenes by splice pathway alteration. Recently, has been demonstrated that CRISPR/Cas9-mediated alterations in exon boundaries may also result in altered splicing of the respective pre-mRNA, most likely due to mutations of splice-regulatory sequences. Using the human FLOT-1 gene as an example, the authors demonstrated that such altered splicing products also give rise to aberrant protein products with loss-of-function [63].

In another unpublished study has been compared the efficiency of generating null alleles by CRISPR/Cas9 sgRNAs targeting exon boundaries. In that study, the authors compared the efficiency of producing null alleles inducing DSBs in a central position of the critical exon versus DSBs close the splice donor site on the exon. The study carried out in different genes, species and systems shows an increase of knock-out efficiency using sgRNA guides targeting the splice-donor site of the elected exon.

### **Conclusion(s)**

Genome editing nucleases like the popular CRISPR/Cas9 allow generate knock-out cell lines and nulls zygotes by inducing site-specific DSB within a genome. In most cases, when a DNA template is not present, the DSB is repaired by non-homologous end joining (NHEJ) resulting in small nucleotide insertions or deletions that can be used to construct knockout alleles. However, for several reasons, these mutations do not produce the desired null result in all cases, generating a

similar protein with functional activity. That undesirable effect could limit the therapeutic efficiency of gene therapy strategies focused on abrogating oncogene expression by CRISPR/Cas9 and should be taken in account. The use of a sgRNAs targeting splicing site could improve the null result for *in vivo* gene therapies. This strategy could be adopted to *in vivo* abrogate the oncogenic effect involved for tumor maintenance.

## Acknowledgments

The authors wish to express their sincere thanks to Dionisio Martín, Alberto Pendás (Spanish Research Rouncil –CSIC-), Lucía Méndez, María Herrero, Sandra Ramos, Elena Vuelta and Laura Lozano (University of Salamanca) for their technical assistance and contribution in our CRISPR/Cas9 studies.

## Conflict of Interest

The authors declare that they have no conflict of interest.

## FUNDING

Our CRISPR/Cas9 studies are supported in part by a grant from the Fondo de Investigaciones Sanitarias (FIS) of the Spanish Ministry of Economy (PI17/01895), Novartis grant and by a Junta de Castilla y León, Fondos FEDER (SA085U16).

## References

1. Ginn SL, Amaya AK, Alexander IE, Edelstein M, Abedi MR. Gene therapy clinical trials worldwide to 2017: An update. *The journal of gene medicine*. 2018:e3015.
2. Walther W, Schlag PM. Current status of gene therapy for cancer. *Current opinion in oncology*. 2013;25(6):659-64.
3. Sridharan K, Gogtay NJ. Therapeutic nucleic acids: current clinical status. *British journal of clinical pharmacology*. 2016;82(3):659-72.
4. Ankathil R, Azlan H, Dzarr AA, Baba AA. Pharmacogenetics and the treatment of chronic myeloid leukemia: how relevant clinically? An update. *Pharmacogenomics*. 2018;19(5):475-393.
5. Graham SM, Jorgensen HG, Allan E, Pearson C, Alcorn MJ, Richmond L, et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 *in vitro*. *Blood*. 2002;99(1):319-25.
6. Duda DG, Kozin SV, Kirkpatrick ND, Xu L, Fukumura D, Jain RK. CXCL12 (SDF1 $\alpha$ )-CXCR4/CXCR7 pathway inhibition: an emerging sensitizer for anticancer therapies? *Clin Cancer Res*. 2011;17(8):2074-80.
7. Nakhband A, Barar J, Bidmeshkipour A, Heidari HR, Omid Y. Bioimpacts of anti epidermal growth factor receptor antisense complexed with polyamidoamine dendrimers in human lung epithelial adenocarcinoma cells. *Journal of biomedical nanotechnology*. 2010;6(4):360-9.
8. Zhao FJ, Zhang SL, Ma L, Gao H, Zong ZH. Inhibitory effects of c-erbB-2 antisense oligonucleotide transfection on uterine endometrial cancer Ishikawa cell lines. *European journal of gynaecological oncology*. 2009;30(1):54-9.

9. Lorient Y, Mordant P, Brown BD, Bourhis J, Soria JC, Deutsch E. Inhibition of BCL-2 in small cell lung cancer cell lines with oblimersen, an antisense BCL-2 oligodeoxynucleotide (ODN): in vitro and in vivo enhancement of radiation response. *Anticancer research*. 2010;30(10):3869-78.
10. Santel A, Aleku M, Roder N, Mopert K, Durieux B, Janke O, et al. Atu027 prevents pulmonary metastasis in experimental and spontaneous mouse metastasis models. *Clin Cancer Res*. 2010;16(22):5469-80.
11. Gaj T, Gersbach CA, Barbas CF, 3rd. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol*. 2013;31(7):397-405.
12. Grimm D, Kay MA. RNAi and gene therapy: a mutual attraction. *Hematology American Society of Hematology Education Program*. 2007:473-81.
13. Bobbin ML, Rossi JJ. RNA Interference (RNAi)-Based Therapeutics: Delivering on the Promise? *Annual review of pharmacology and toxicology*. 2016;56:103-22.
14. Porteus M. Genome Editing: A New Approach to Human Therapeutics. *Annual review of pharmacology and toxicology*. 2016;56:163-90.
15. Garcia-Tunon I, Hernandez-Sanchez M, Ordonez JL, Alonso-Perez V, Alamo-Quijada M, Benito R, et al. The CRISPR/Cas9 system efficiently reverts the tumorigenic ability of BCR/ABL in vitro and in a xenograft model of chronic myeloid leukemia. *Oncotarget*. 2017.
16. Mojica FJM, Montoliu L. On the Origin of CRISPR-Cas Technology: From Prokaryotes to Mammals. *Trends Microbiol*. 2016;24(10):811-20.
17. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 2012;337(6096):816-21.
18. Ceccaldi R, Rondinelli B, D'Andrea AD. Repair Pathway Choices and Consequences at the Double-Strand Break. *Trends in cell biology*. 2016;26(1):52-64.
19. Chiruvella KK, Liang Z, Wilson TE. Repair of double-strand breaks by end joining. *Cold Spring Harbor perspectives in biology*. 2013;5(5):a012757.
20. Karanam K, Kafri R, Loewer A, Lahav G. Quantitative live cell imaging reveals a gradual shift between DNA repair mechanisms and a maximal use of HR in mid S phase. *Mol Cell*. 2012;47(2):320-9.
21. Heyer WD, Ehmsen KT, Liu J. Regulation of homologous recombination in eukaryotes. *Annual review of genetics*. 2010;44:113-39.
22. Simsek D, Jasin M. Alternative end-joining is suppressed by the canonical NHEJ component Xrcc4-ligase IV during chromosomal translocation formation. *Nature structural & molecular biology*. 2010;17(4):410-6.
23. Deriano L, Roth DB. Modernizing the nonhomologous end-joining repertoire: alternative and classical NHEJ share the stage. *Annual review of genetics*. 2013;47:433-55.
24. Wassef M, Luscan A, Battistella A, Le Corre S, Li H, Wallace MR, et al. Versatile and precise gene-targeting strategies for functional studies in mammalian cell lines. *Methods*. 2017;121-122:45-54.
25. Cyranoski D. CRISPR gene-editing tested in a person for the first time. *Nature*. 2016;539(7630):479.
26. Kotterman MA, Schaffer DV. Engineering adeno-associated viruses for clinical gene therapy. *Nat Rev Genet*. 2014;15(7):445-51.
27. Kim E, Koo T, Park SW, Kim D, Kim K, Cho HY, et al. In vivo genome editing with a small Cas9 orthologue derived from *Campylobacter jejuni*. *Nat Commun*. 2017;8:14500.
28. Sanchez-Garcia I. Consequences of chromosomal abnormalities in tumor development. *Annual review of genetics*. 1997;31:429-53.
29. Perez-Mancera PA, Sanchez-Garcia I. Understanding mesenchymal cancer: the liposarcoma-associated FUS-DDIT3 fusion gene as a model. *Seminars in cancer biology*. 2005;15(3):206-14.
30. Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature*. 1973;243(5405):290-3.
31. Rowley JD. Identification of a translocation with quinacrine fluorescence in a patient with acute leukemia. *Annales de genetique*. 1973;16(2):109-12.
32. Ren R. Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. *Nature reviews Cancer*. 2005;5(3):172-83.
33. Palacios R, Henson G, Steinmetz M, McKearn JP. Interleukin-3 supports growth of mouse pre-B-cell clones in vitro. *Nature*. 1984;309(5964):126-31.

34. Gomez HL, Felipe-Medina N, Sanchez-Martin M, Davies OR, Ramos I, Garcia-Tunon I, et al. C14ORF39/SIX6OS1 is a constituent of the synaptonemal complex and is essential for mouse fertility. *Nat Commun.* 2016;7:13298.
35. Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, et al. In vivo genome editing using *Staphylococcus aureus* Cas9. *Nature.* 2015;520(7546):186-91.
36. Kouranova E, Forbes K, Zhao G, Warren J, Bartels A, Wu Y, et al. CRISPRs for Optimal Targeting: Delivery of CRISPR Components as DNA, RNA, and Protein into Cultured Cells and Single-Cell Embryos. *Human gene therapy.* 2016;27(6):464-75.
37. Lau CH, Suh Y. In vivo genome editing in animals using AAV-CRISPR system: applications to translational research of human disease. *F1000Research.* 2017;6:2153.
38. Williams MR, Fricano-Kugler CJ, Getz SA, Skelton PD, Lee J, Rizzuto CP, et al. A Retroviral CRISPR-Cas9 System for Cellular Autism-Associated Phenotype Discovery in Developing Neurons. *Sci Rep.* 2016;6:25611.
39. Koike-Yusa H, Li Y, Tan EP, Velasco-Herrera Mdel C, Yusa K. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat Biotechnol.* 2014;32(3):267-73.
40. Kabadi AM, Ousterout DG, Hilton IB, Gersbach CA. Multiplex CRISPR/Cas9-based genome engineering from a single lentiviral vector. *Nucleic Acids Res.* 2014;42(19):e147.
41. Wang D, Mou H, Li S, Li Y, Hough S, Tran K, et al. Adenovirus-Mediated Somatic Genome Editing of Pten by CRISPR/Cas9 in Mouse Liver in Spite of Cas9-Specific Immune Responses. *Human gene therapy.* 2015;26(7):432-42.
42. Cheng R, Peng J, Yan Y, Cao P, Wang J, Qiu C, et al. Efficient gene editing in adult mouse livers via adenoviral delivery of CRISPR/Cas9. *FEBS Lett.* 2014;588(21):3954-8.
43. Schmidt F, Grimm D. CRISPR genome engineering and viral gene delivery: a case of mutual attraction. *Biotechnology journal.* 2015;10(2):258-72.
44. Swiech L, Heidenreich M, Banerjee A, Habib N, Li Y, Trombetta J, et al. In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. *Nat Biotechnol.* 2015;33(1):102-6.
45. Mansouri M, Ehsaei Z, Taylor V, Berger P. Baculovirus-based genome editing in primary cells. *Plasmid.* 2017;90:5-9.
46. Mansouri M, Bellon-Echeverria I, Rizk A, Ehsaei Z, Cianciolo Cosentino C, Silva CS, et al. Highly efficient baculovirus-mediated multigene delivery in primary cells. *Nat Commun.* 2016;7:11529.
47. Chen X, Goncalves MA. Engineered Viruses as Genome Editing Devices. *Mol Ther.* 2016;24(3):447-57.
48. Kwang TW, Zeng X, Wang S. Manufacturing of AcMNPV baculovirus vectors to enable gene therapy trials. *Molecular therapy Methods & clinical development.* 2016;3:15050.
49. Epstein BE, Schaffer DV. Combining Engineered Nucleases with Adeno-associated Viral Vectors for Therapeutic Gene Editing. *Advances in experimental medicine and biology.* 2017;1016:29-42.
50. Kennedy EM, Cullen BR. Gene Editing: A New Tool for Viral Disease. *Annual review of medicine.* 2017;68:401-11.
51. Friedland AE, Baral R, Singhal P, Loveluck K, Shen S, Sanchez M, et al. Characterization of *Staphylococcus aureus* Cas9: a smaller Cas9 for all-in-one adeno-associated virus delivery and paired nickase applications. *Genome biology.* 2015;16:257.
52. Kay MA. State-of-the-art gene-based therapies: the road ahead. *Nat Rev Genet.* 2011;12(5):316-28.
53. Zincarelli C, Soltys S, Rengo G, Rabinowitz JE. Analysis of AAV serotypes 1-9 mediated gene expression and tropism in mice after systemic injection. *Mol Ther.* 2008;16(6):1073-80.
54. Smith LJ, Ul-Hasan T, Carvaines SK, Van Vliet K, Yang E, Wong KK, Jr., et al. Gene transfer properties and structural modeling of human stem cell-derived AAV. *Mol Ther.* 2014;22(9):1625-34.
55. Krawczak M, Reiss J, Cooper DN. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Human genetics.* 1992;90(1-2):41-54.
56. Treisman R, Orkin SH, Maniatis T. Specific transcription and RNA splicing defects in five cloned beta-thalassaemia genes. *Nature.* 1983;302(5909):591-6.
57. Treisman R, Proudfoot NJ, Shander M, Maniatis T. A single-base change at a splice site in a beta 0-thalassaemic gene causes abnormal RNA splicing. *Cell.* 1982;29(3):903-11.

58. Camats N, Pandey AV, Fernandez-Cancio M, Fernandez JM, Ortega AM, Udhane S, et al. STAR splicing mutations cause the severe phenotype of lipid congenital adrenal hyperplasia: insights from a novel splice mutation and review of reported cases. *Clin Endocrinol (Oxf)*. 2014;80(2):191-9.
59. Matsuda D, Sato H, Maquat LE. Chapter 9. Studying nonsense-mediated mRNA decay in mammalian cells. *Methods in enzymology*. 2008;449:177-201.
60. Elsayed LEO, Mohammed IN, Hamed AAA, Elseed MA, Salih MAM, Yahia A, et al. Case report of a novel homozygous splice site mutation in PLA2G6 gene causing infantile neuroaxonal dystrophy in a Sudanese family. *BMC medical genetics*. 2018;19(1):72.
61. Liu G, Wang X, Qin M, Sun L, Zhu J. A Novel Splicing Mutation of Ectodysplasin A Gene Responsible for Hypohidrotic Ectodermal Dysplasia. *Oral diseases*. 2018.
62. Wood-Trageser MA, Gurbuz F, Yatsenko SA, Jeffries EP, Kotan LD, Surti U, et al. MCM9 mutations are associated with ovarian failure, short stature, and chromosomal instability. *American journal of human genetics*. 2014;95(6):754-62.
63. Kapahnke M, Banning A, Tikkanen R. Random Splicing of Several Exons Caused by a Single Base Change in the Target Exon of CRISPR/Cas9 Mediated Gene Knockout. *Cells*. 2016;5(4).
64. Gomez-H L, Felipe-Medina N, Sanchez-Martin M, Davies OR, Ramos I, Garcia-Tunon I, et al. C14ORF39/SIX6OS1 is a constituent of the synaptonemal complex and is essential for mouse fertility. *Nature Communications*. 2016;7.

### Citing Sources

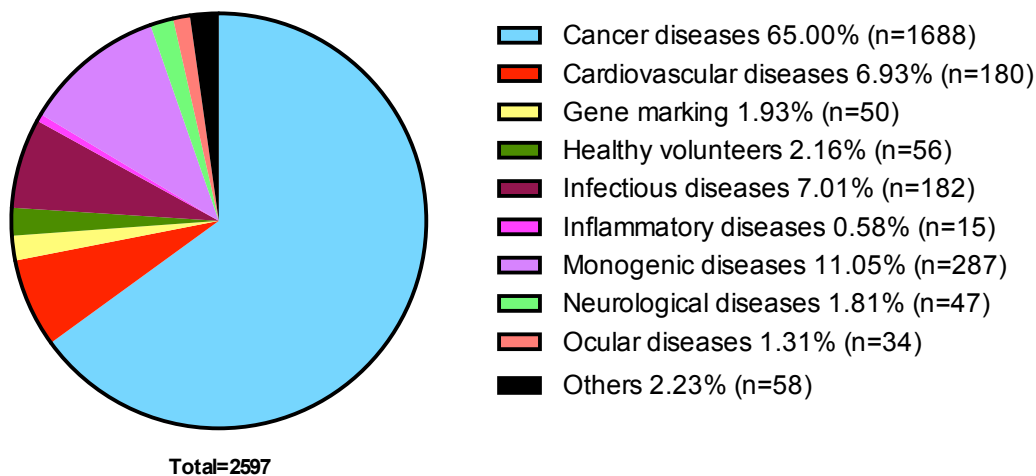
When you are **citing sources**, the citations should be set in numbered format. All the references given in the list of references should be cited in the body of the text. Please set citations in square brackets keeping the below points in mind:

Correct format: [4–6, 9]; [1, 2]

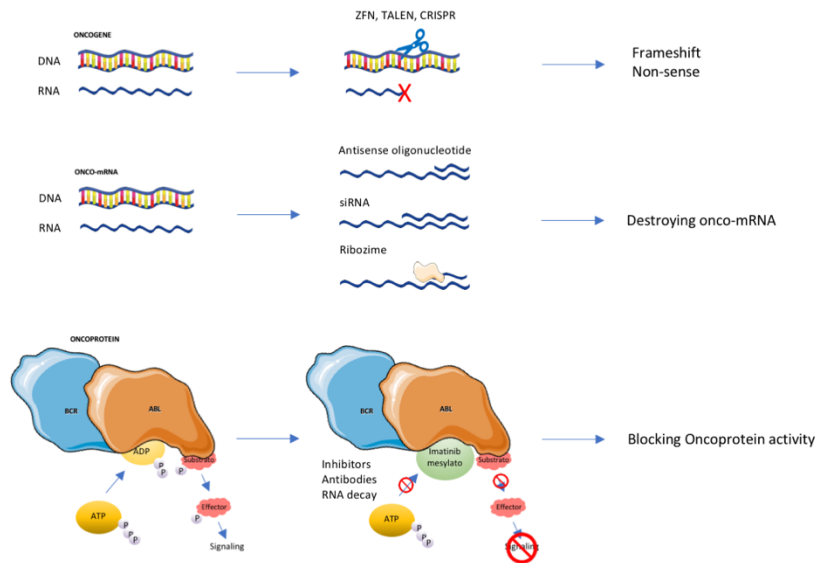
Incorrect format: [4-6,9], [4, 5, 6, 9], [4] [5] [6] [9]; [1–2]

The numbers should be listed in sequential order.

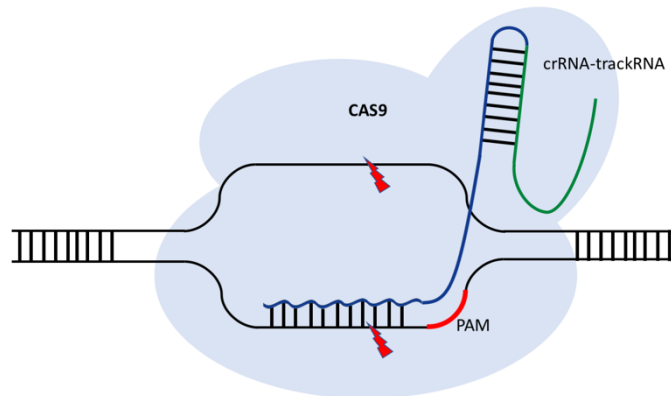
### Figures and Tables



**Figure 1.- Gene therapy trials worldwide**

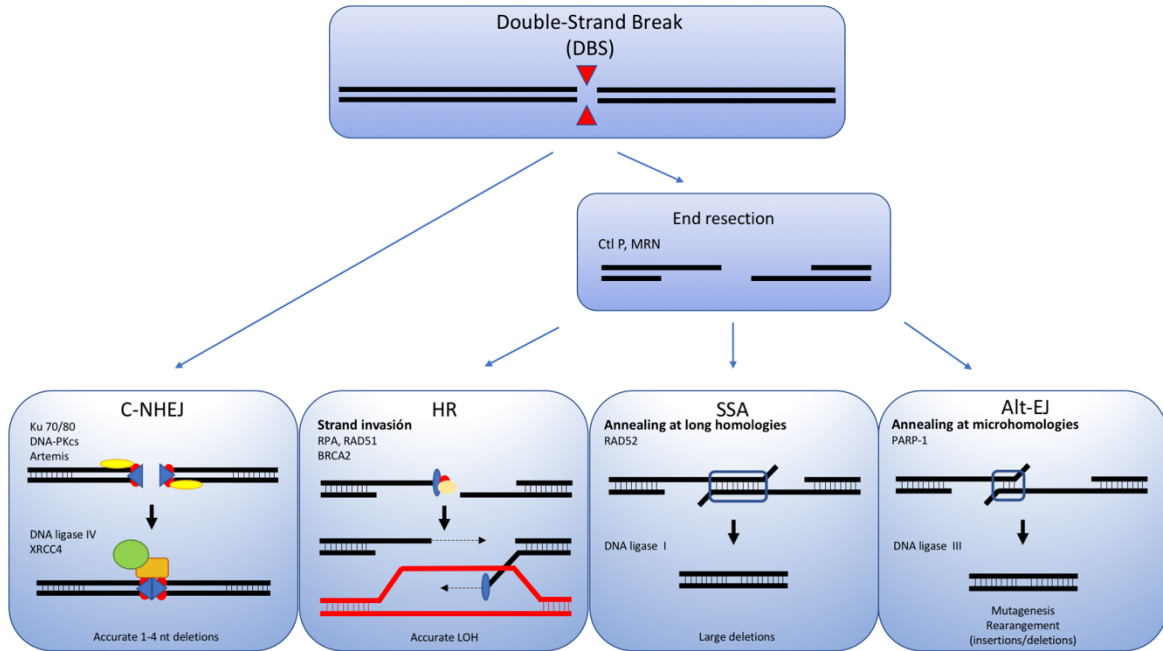


**Figure 2.- Different strategies to block oncogene effects.**

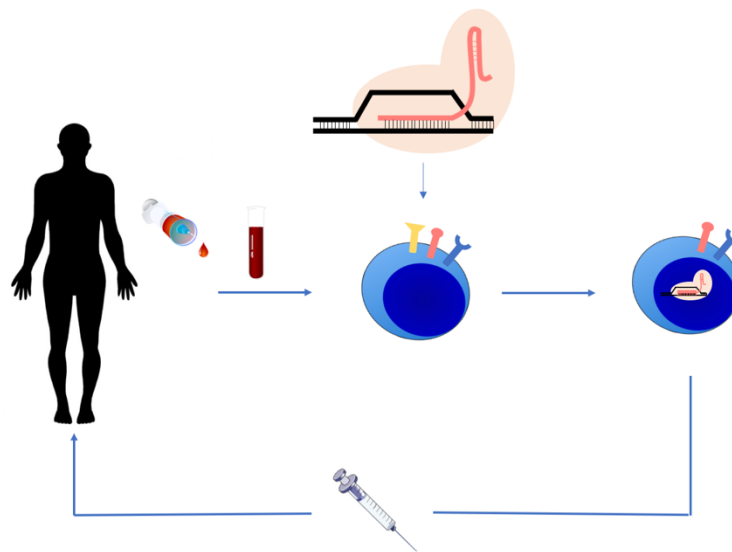


**Figure 3.- CRISPR/Cas9 Ribonucleoprotein complex.** Cas9 nuclease is driven to the target DNA sequence by a sgRNA molecule, composed by crRNA (blue) and trackRNA (green). Target sequence must be followed immediately by Protospacer adjacent motif sequence (PAM). After hybridization of 20 nt of the crRNA with the target sequence, the nuclease performs a double-stranded break 3 nt upstream from the PAM sequence.

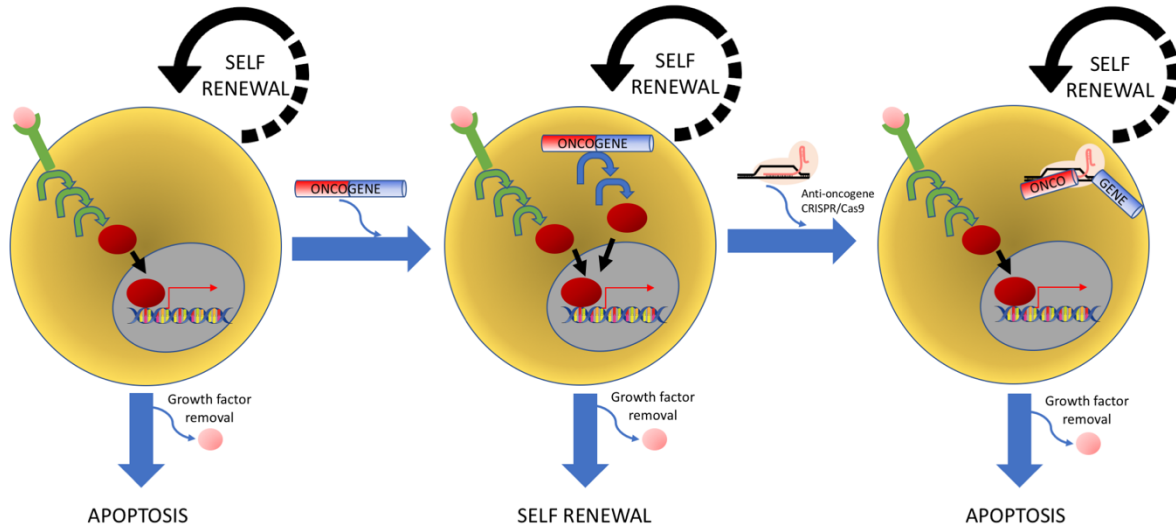




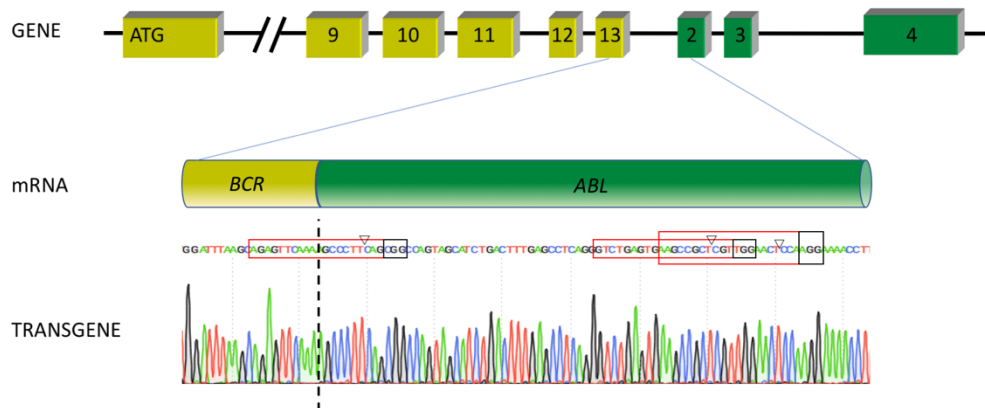
**Figure 4.-Approaches to repair DNA double-Strand Breakes.** When DNA resection is blocked, C-NHEJ (classical nonhomologous end joined) is well-established, whereas if the resection occurs, DNA damage is repaired by HR (homologous recombination), SSA (single-strand annealing) or alt-EJ (alternative end joining) [18].



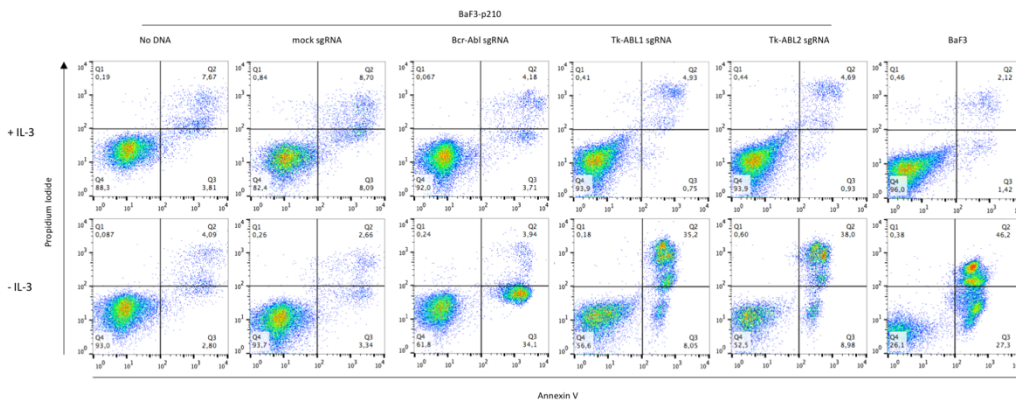
**Figure 5.- Experimental design of the first CRISPR/Cas9 edited cells injection in humans.** Immune precursor cells were isolated from blood, and in vitro CRISPR/Cas9 edited to eliminate PD-1 gene. Then, modified cell were reinfused into patient [25].



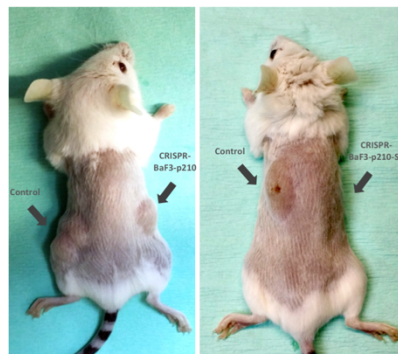
**Figure 6.- Experimental model to show CRISPR/Cas9 ability to truncate BCR/ABL fusion.** Non-tumorigenic and IL-3 dependent BaF3 cell line was transformed with fusion oncogene BCR/ABLp210. Transformed cell line is able to growth and survive in absence of IL-3, however, the cells enter into apoptosis when CRISPR/Cas9 introduces mutations in the sequence of the BCR/ABL oncogene, preventing its expression [15].



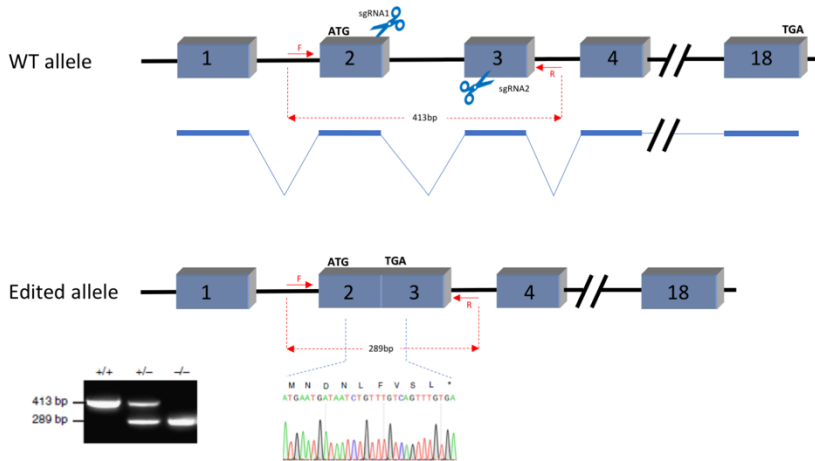
**Figure 7.- Schematic representation of BCR/ABL fusion transgene.** Sequences of sgRNAs designed to edit fusion region (red boxes), one of them hybridize in the junction BCR/ABL and the other two in the exon 2 of ABL [15].



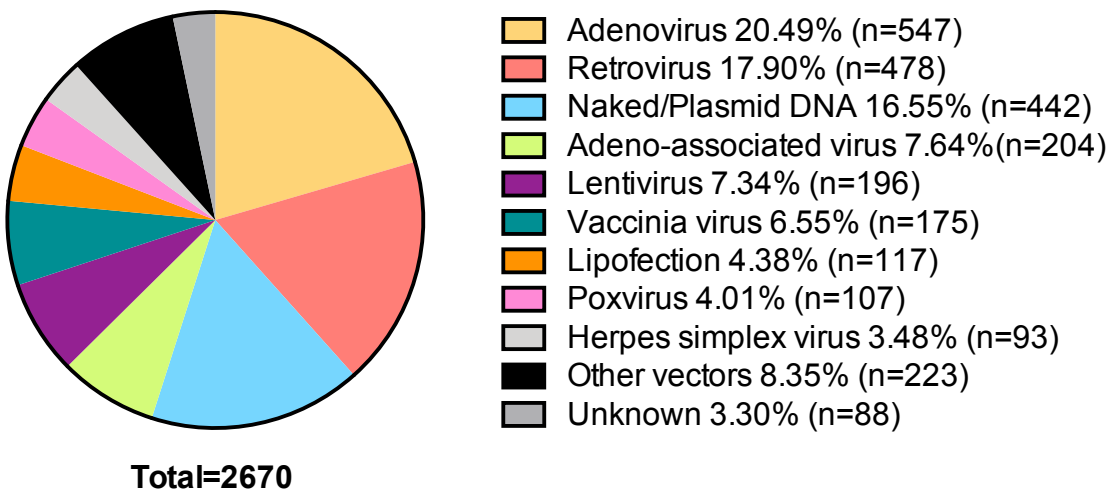
**Figure 8.- Functional analysis of CRISPR-BaF3-p210 edited cells.** Annexin V labelling was measured by flow cytometry in edited cells (BCR-ABL, TK-ABL1 and TK-ABL-2) in presence and absence of IL-3. When the IL-3 was removed from the medium, the 3 cell lines published showed an increase in apoptosis, a reflection of the absence of expression of the BCR-ABL oncogene [15].



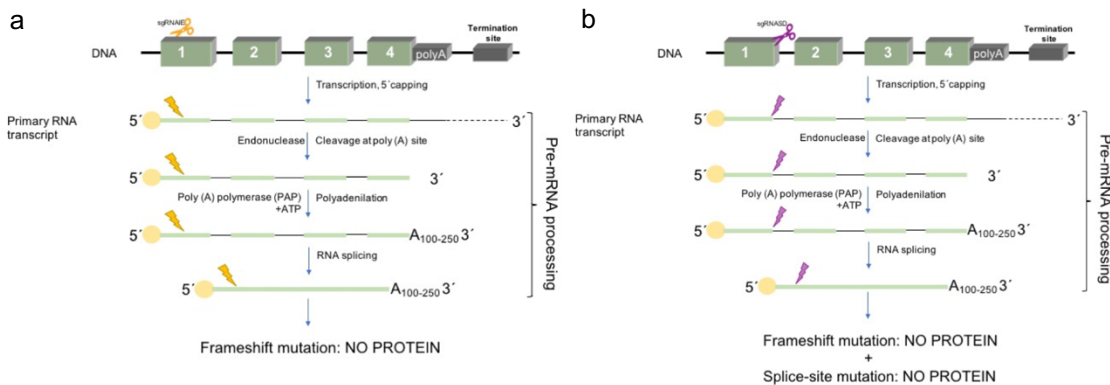
**Figure 9.- In Vivo effects of CRISPR-mediated editing of BCR/ABL oncogene.** Tumor growth over 24 days following subcutaneous cell injection. The final tumor mass was reduced by half in the CRISPR-BaF3-p210, relative to controls. CRISPR-BaF3-Single Cell derived cell line (SC) cells were unable to form a subcutaneous tumor [15].



**Figure 10.- Schematic representation of Six6os1 WT and edited allele.** Two sgRNA were used to produce a deletion between exon 2 and 3. As a consequence, a premature stop codon appears at the beginning of exon 3. Edited allele can be easily detectable by PCR [64].



**Figure 11.- Delivery systems commonly used in gene therapy clinical trials**



**Figure 12.- CRISPR/Cas9 design against sequences involved in the intron processing.**

Indications	Gene therapy clinical trials	
	Number	%
Cancer diseases	1688	65
Cardiovascular diseases	180	6.9
Gene marking	50	1.9
Healthy volunteers	56	2.2
Infectious diseases	182	7
Inflammatory diseases	15	0.6
Monogenic diseases	287	11.1
Neurological diseases	47	1.8
Ocular diseases	34	1.3
Others	58	2.2

**Table 1.- Gene therapy trials worldwide**

Guide	Edition efficiency	Tide predicted indels
Bcr-Abl sgRNA	85 %	+1bp (17.5%), -1bp (9.1%), -2bp (4.8%) -3bp (3.4%), -4bp (6%), -6bp (1.8%), -8bp (18.9%), -11bp (10.2%), -18bp (5.1%)
TK-ABL 1 sgRNA	54,6 %	+1bp (14.9%), -1bp (8%) -2bp (5.2%), -10bp (17.6%)
TK-ABL 2 sgRNA	68,8 %	+1bp (30.8%), -1bp (5.9%), -2bp (4.8%), -4bp (15.2%), -14bp (5.1%)
Mock sgRNA	0%	

**Table 2.- TIDE algorithm predicted indels induced by each sgRNA**

Characteristics for a typical vector	Retrovirus	Lentivirus	Adenovirus	Adeno-associated virus	Baculovirus
Common viral type	γ-retroviruses	HIV-1	Ad5	AAV2	AcMNPV
Viral genome structure	Linear ssRNA	Linear ssRNA	Linear dsDNA	Linear ssDNA	Circular dsDNA
Viral genome size	8.3 kb	9.7 kb	36 kb	4.7 kb	80–180 kb
Packaging capacity	<8.0 kb	<8.0 kb	<30 kb	<4.5 kb	>38 kb
Cells infected	Dividing	Dividing or non-dividing	Dividing or non-dividing	Dividing or non-dividing	Dividing or non-dividing
Transduction efficiency	Moderate	High	Very high	High	High
Transgene expression	Stable	Stable	Transient	Transient	Transient
Immune response	Moderate	Low	High	Very low	Very low
Toxicity	High	Moderate	High	Low	Low
Random genome integration	Yes	Yes	No	Generally, no (recombinant AAV has a low	No

				frequency of host genome integration events)	
Common applications	Generating stable cell and gene transfer, cancer and stem cell research	Transduce difficult-to-transfect cell, genome-wide screens	Vaccine production, cancer immune therapy	Gene delivery <i>in vivo</i> , optogenetics	Recombinant proteins and vaccine production
Clinical trials	Very popular	Very popular	Popular	Increasing popularity	Growing interest

**Table 3.- Most used viral delivery system in gene therapy**