Chapter 2

CRISPR-ERA for Switching Off (Onco) Genes

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http://dx.doi.org/10.5772/intechopen.80245

Abstract

Genome-editing nucleases like the popular CRISPR/Cas9 enable the generation of knockout cell lines and null zygotes by inducing site-specific double-stranded breaks (DSBs) within a genome. In most cases, when a DNA template is not present, the DSB is repaired by nonhomologous 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, instead generating a similar protein with functional activity. This undesirable effect could limit the therapeutic efficiency of gene therapy strategies focused on abrogating oncogene expression by CRISPR/Cas9 and should be taken into account. This chapter reviews the irruption of CRISPR technology for gene silencing and its application in gene therapy.

Keywords: gene therapy, knockout, null allele, oncogene silencing, CRISPR technology, gene suppression

1. Gene suppression therapies in cancer: an overview

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

Various strategies at different molecular levels (**Figure 2**) have been employed to treat malignant diseases in recent decades, such as specific drug inhibitors acting at the protein level,

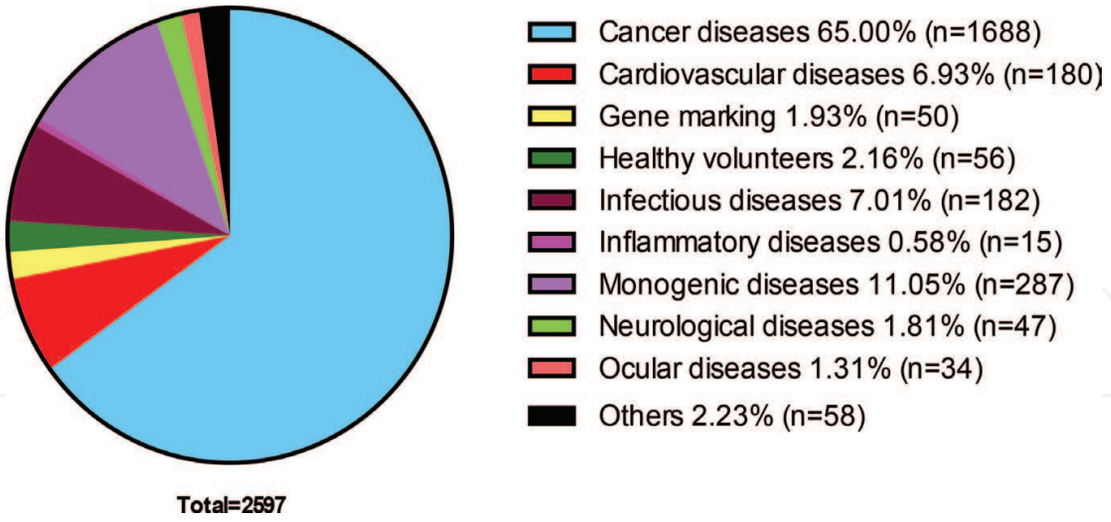

Figure 1. Gene therapy trials worldwide.

Indications	Gene therapy clinical trials	
	Number	%
Cancer diseases	1688	65.0
Cardiovascular diseases	180	6.9
Gene marking	50	1.9
Healthy volunteers	56	2.2
Infectious diseases	182	7.0
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.

gene suppression therapies at the mRNA level, and genome-editing nucleases at the 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 being imatinib mesylate, a tyrosine-kinase inhibitor (TKI) indicated for the first-line treatment of chronic myeloid leukemia (CML). The advent of imatinib mesylate at the end of the twentieth century has revolutionized CML prognosis, yielding an overall survival (OS) rate of 88% after 5 years, whereas previous nonspecific treatments produced an OS rate of only 57% [4]. Unfortunately, despite the increased efficacy and better clinical responses, many patients receiving targeted drugs have a poor initial response, develop resistance, or undergo relapse after initial success. Except for a subgroup of patients who achieve a deep and sustained molecular response, TKI

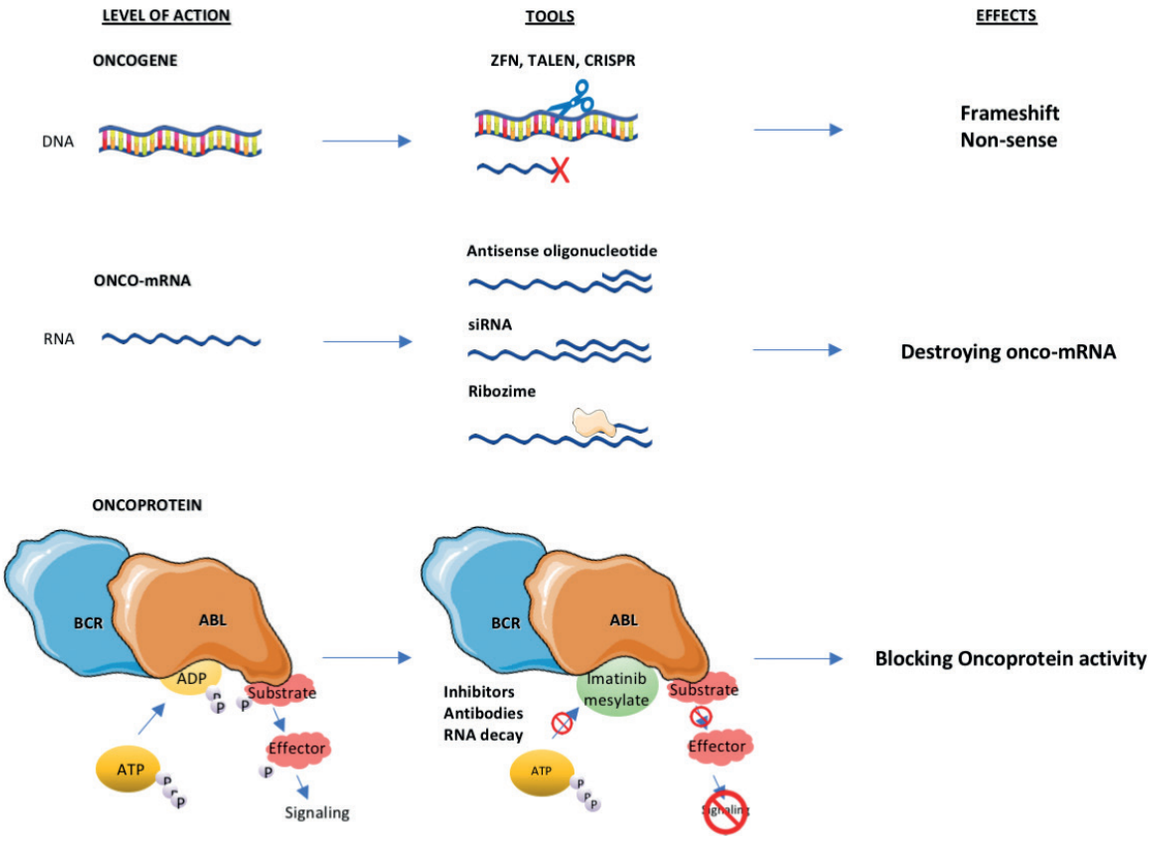

Figure 2. Different strategies to block oncogene effects.

therapies would need to be continued indefinitely because TKIs do not completely eliminate the leukemia stem cells (LSCs), but they remain even during effective TKI treatment [5].

An alternative oncoprotein inhibition approach emerges from the ability of some small RNAs to fold into three-dimensional structures that 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 regulatory chemokine essential for the migration of leukemic stem cells into the bone marrow [6]. This inhibition of the binding of SDF-1 to its receptors can prompt the leukemic stem cells to re-enter the cell cycle and become vulnerable to chemotherapeutic attack.

Other gene suppression therapies focus on the intervention of gene transcription and translation, which are 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 be applied in cancer and virology. Transcripts of genes of different function have been targeted by AS-ODN gene therapies such as c-myb, 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 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 been successful at inhibiting tumor progression and metastasis in lung and mammary carcinoma models [10]. Nonetheless, inefficient/complete silencing and transient effects present major challenges to cancer gene therapy mediated by ribozymes, AS-ODNs, or siRNAs [2]. Other important challenges that need to be addressed for the successful translation of these approaches are their delivery to the site of action, the choice between direct delivery or the use of a vehicle, mass production at low cost, more clearly defined pharmacokinetics, and the 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 their nuclease-associated protein Cas9 (CRISPR/Cas9), the landscape of 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 with which these reagents can be designed and tested through the construction of single-guide RNAs (sgRNAs) has made gene editing available to a wider variety of users and for a broader range of applications.

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

2. CRISPR-Cas9 technology, DSBs, and gene interruption

The CRISPR/Cas9 system allows sequence-specific gene editing in many organisms and is currently the best tool for generating cell lines and animal models of human diseases. The main advantages of this technology are its simplicity, versatility, and efficiency compared with other gene-modifying technologies. CRISPR/Cas9 technology is usually used to introduce targeted 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′ to the targeted DNA sequence [17] (**Figure 3**).

Following the creation of a 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 standard 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 G0/G1

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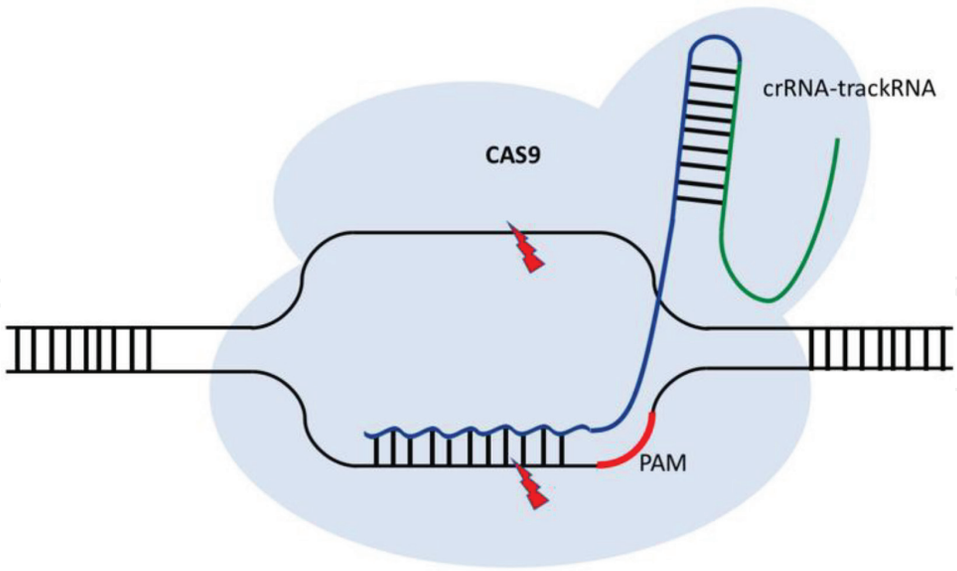

Figure 3. CRISPR/Cas9 ribonucleoprotein complex. Cas9 nuclease is driven to the target DNA sequence by an sgRNA molecule, composed by crRNA (blue) and trackRNA (green). The target sequence must be followed immediately by a protospacer adjacent motif sequence (PAM). After hybridization of 20 nt of the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

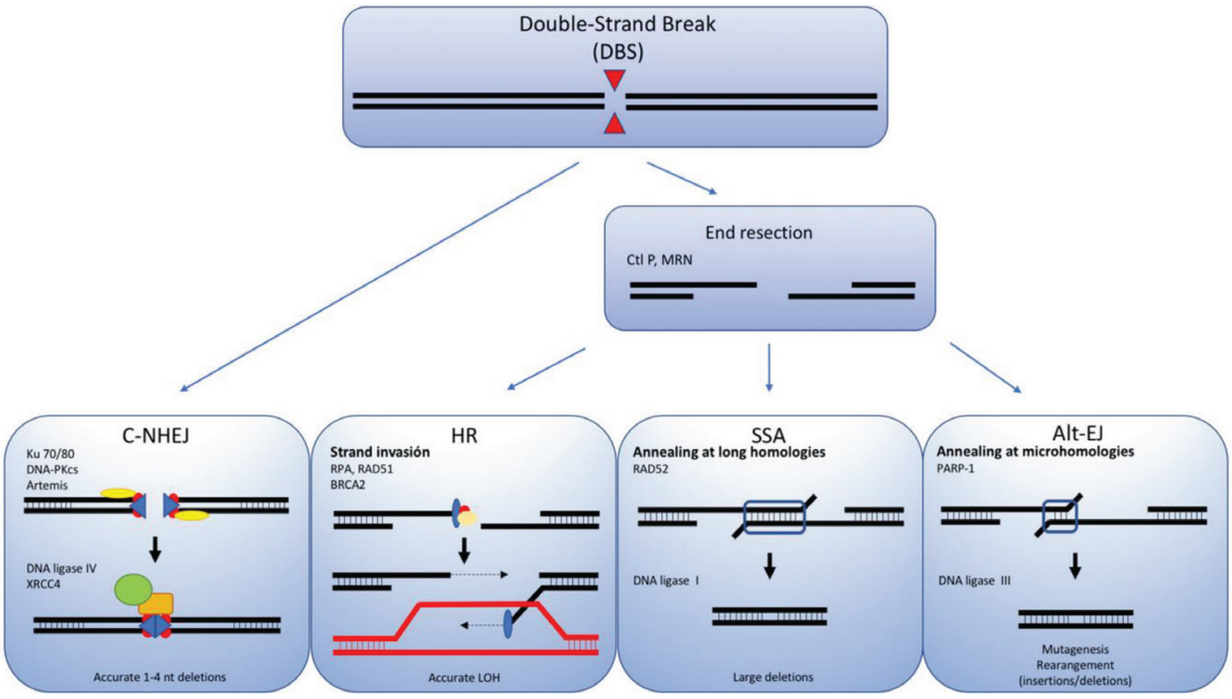

Figure 4. Approaches to repair DNA double-strand breaks. When DNA resection is blocked, C-NHEJ (classic nonhomologous end joining) is well established, whereas if the resection does occur, DNA damage is repaired by HR (homologous recombination), SSA (single-strand annealing) or alt-EJ (alternative end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

and G2 and is associated with 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-G2 cell cycle phases, where the amount of DNA replication is highest and when the sister template is available [20]. HR uses a template for repair and so requires strand invasion mediated by the recombinase RAD51 (**Figure 4**) [21]. It may be possible to exploit this property to edit mutations, delivering the appropriate template joined to the CRISPR/Cas9 system inside the target 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]. The alt-EJ mechanism is not well understood but has an apparent predilection for joining DSBs on different chromosomes, thereby 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 a consequence of its efficiency at inducing DSB, CRISPR/Cas9 technology has gained a reputation as the "gold standard" for creating null alleles in both *in vivo* and *in vitro*. These null alleles can arise from frameshift mutations, premature stop codons, and/or non-sense-mediated decay on the target gene, resulting in loss of function. Currently, CRISPR/Cas9 is extensively used to engineer gene knockouts, but due to the variable size of the NHEJ-induced indel, generating a full KO in one step is not always achieved. In fact, full KO generation requires off-frame mutations in both alleles, and this is a matter of probability because several mutations could preserve the reading frame (e.g., +3 or −3 mutations). This undesirable effect

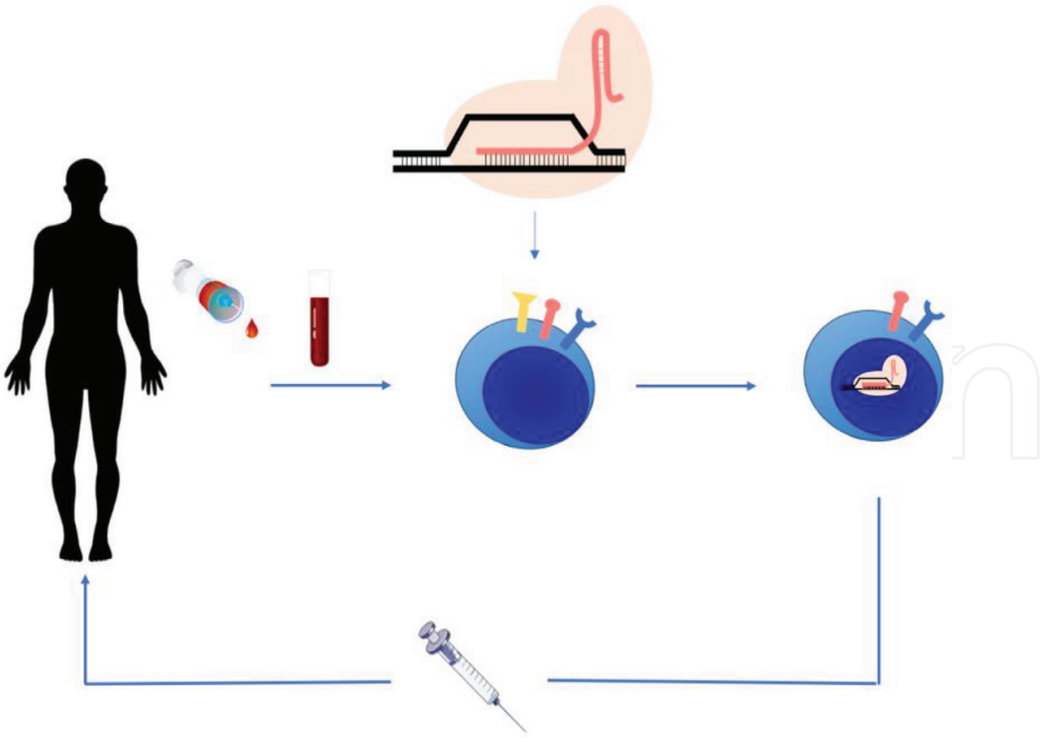

Figure 5. Experimental design of the first CRISPR/Cas9-edited cell injection in humans. Immune precursor cells were isolated from blood and *in vitro* CRISPR/Cas9 edited to eliminate PD-1 gene. Modified cells were then reinfused into the patient [25].

may be irrelevant in assays in which the knockout cell can be selected, or the null allele of the animal model can be segregated [24]. The first clinical trial using CRISPR for gene suppression and cancer therapy enrolled its 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* was evaluated when treating metastatic non–small cell lung cancer that had progressed after employing all standard treatments. Patients enrolled in the gene-editing trial provided peripheral blood lymphocytes, and PD-1 knockout of T-cells by CRISPR/Cas9 was performed *ex vivo*. In this trial, the edited lymphocytes were 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 are not an option, and obtaining 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 this pathological cell context, the highly efficient interruption of the 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 is the case for several leukemias or sarcomas, which are directed by specific fusion oncoproteins [28, 29].

3. CRISPR-Cas9 technology for disrupting fusion oncogenes

Fusion oncoproteins arising from chromosomal rearrangements are known to drive the pathogenesis of a variety of hematological neoplasms such as CML, which results 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 a *BCR/ABL* fusion gene, whose 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 fail to respond adequately to these drugs [4]. A recent study of the BCR/ABL oncogen showed this gene fusion to be an ideal target for CRISPR/Cas9-mediated gene therapy. A CRISPR-Cas9 application truncated the specific BCR-ABL fusion (p210) in an *in vitro* cellular model [15] (**Figure 6**).

In this study, a nontumorigenic cell line (BaF3), which needs IL-3 to survive and proliferate [33], was transformed with the fusion oncogene BCR/ABLp210 (BaF3-p210). The human BCR/ABL oncogenic fusion confers on 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 point in each one.

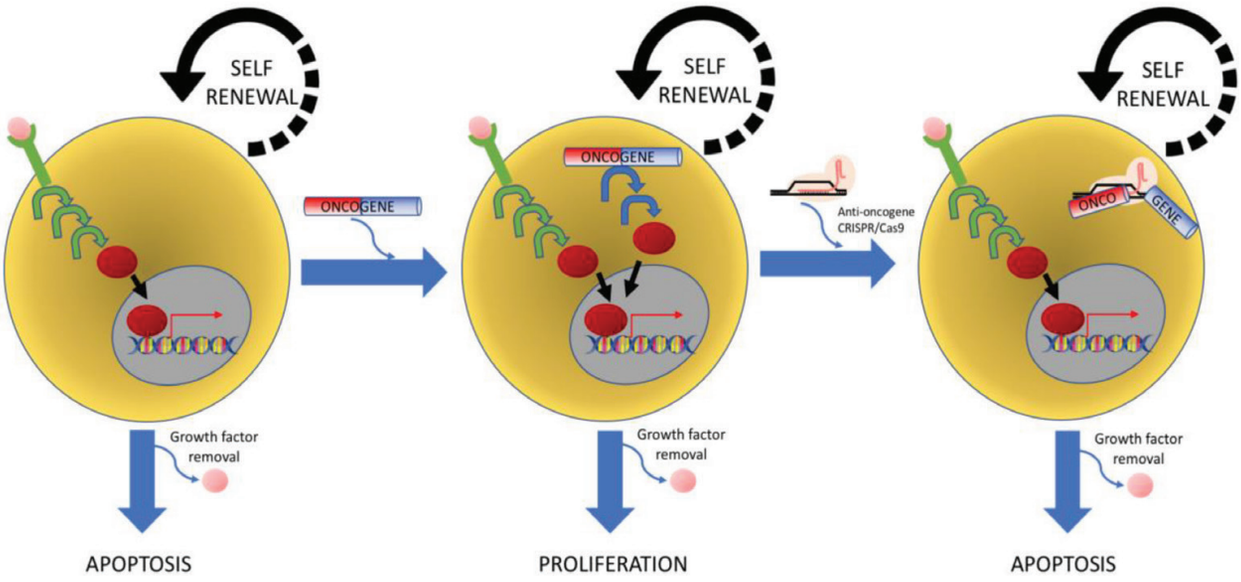

Figure 6. Experimental model to show the ability of CRISPR/Cas9 to truncate BCR/ABL fusion. The non-tumorigenic and IL-3-dependent BaF3 cell line was transformed with fusion oncogene BCR/ABLp210. The transformed cell line is able to grow and survive in the absence of IL-3, although the cells enter into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

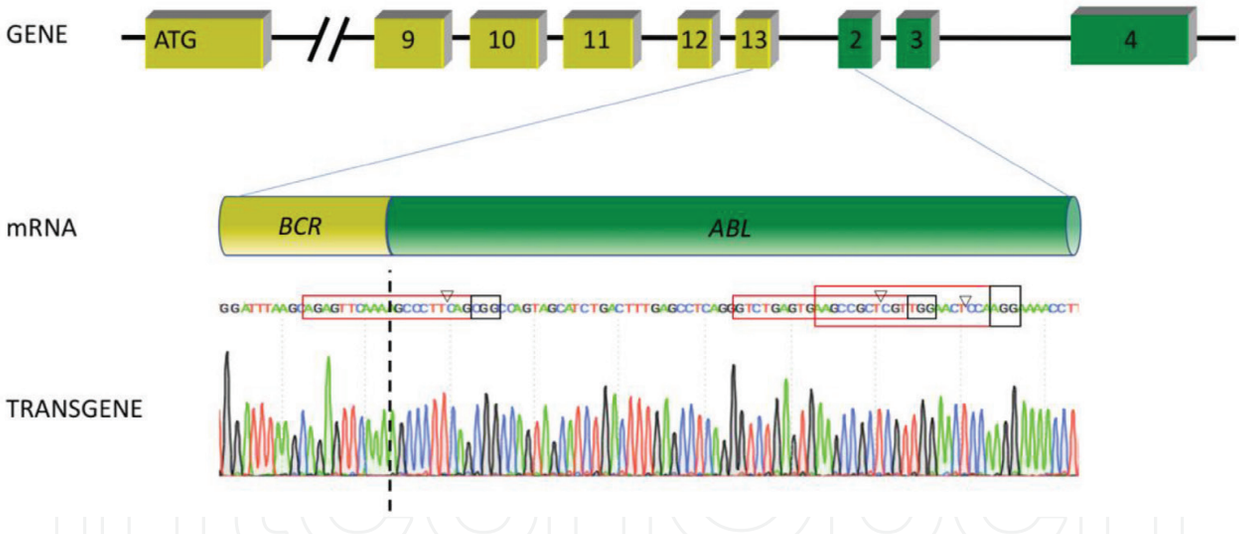

Figure 7. Schematic representation of BCR/ABL fusion transgene. Sequences of sgRNAs designed to edit fusion region (red boxes). One of them hybridizes at the BCR/ABL junction, and the other two hybridize in exon 2 of ABL [15].

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

As a result, significantly more cell death was observed in all CRISPR-BaF3-p210 cell lines in the absence of IL-3 than in BaF3 parental cells or mock BaF3-p210 cells (**Figure 8**).

Xenograft experiments were carried out to determine whether the tumorigenic capacity was also blocked by the action of the CRISPR/Cas9 system. Mice injected with the three

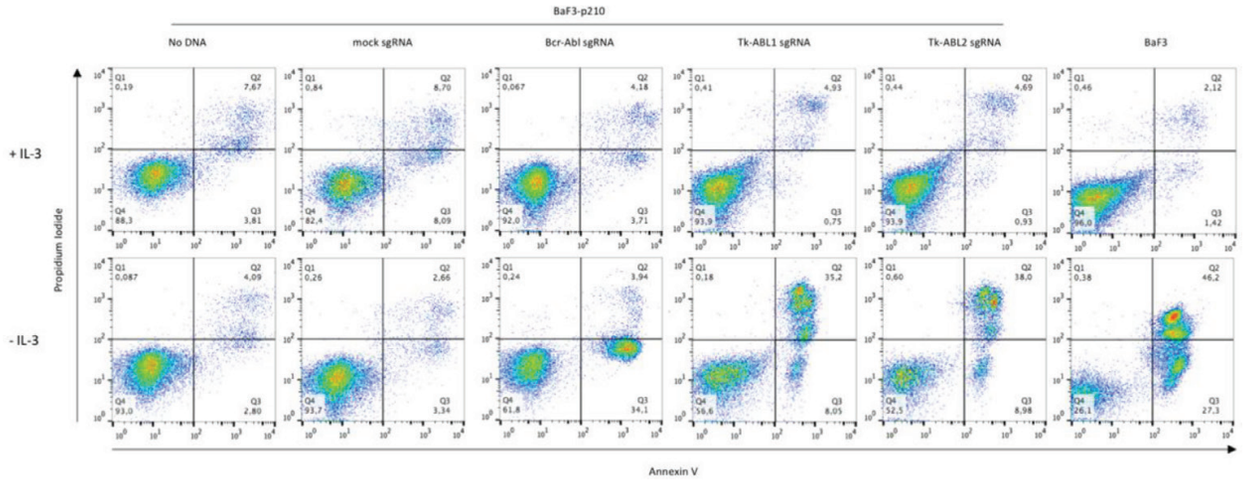

Figure 8. Functional analysis of CRISPR-BaF3-p210-edited cells. Annexin V labeling was measured by flow cytometry in edited cells (BCR-ABL, TK-ABL1 and TK-ABL-2) in the presence and absence of IL-3. When IL-3 was removed from the medium, the three cell lines showed an increase in apoptosis, reflecting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

CRISPR-BaF3-p210 cell lines gave rise to significantly smaller subcutaneous tumors than those produced by the nonedited cells (**Figure 9**).

As expected, these small tumors were composed of nonedited cells, edited cells with +3/−3 bp indels (or multiples), or cells with nonframeshift mutations. This result indicated that a specific cellular selection or more specific sgRNAs should be necessary before potential gene

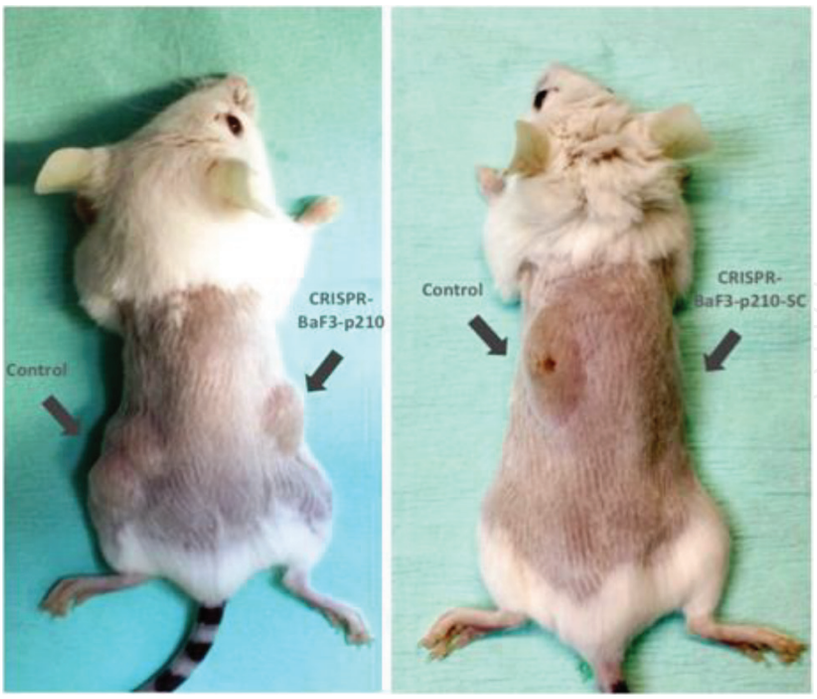

Figure 9. *In vivo* effects of CRISPR-mediated editing of BCR/ABL oncogene. Tumor growth over 24 days following subcutaneous cell injection. The final tumor mass was reduced by half in the CRISPR-BaF3-p210, relative to controls. CRISPR-BaF3 single cell-derived cell line (SC) cells were unable to form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

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 tumorigenic capacity. No tumor growth was observed in any mouse injected with cells derived from the single-edited cell line (**Figure 9**).

3.1. CRISPR/CAS9 and knocking out genes in mouse

An option to improve knockout effectiveness could be to use two or more RNA guides at the same time to knock out the oncogene allele at different key sites in an attempt to try to guarantee the null result. This approach is commonly used for knocking out genes in animal models such as mice. Using two sgRNA guides makes it possible to 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 used. The main difficulty stems from the limitations on the construct, for which reason other Cas9 orthologues are being used to introduce the nuclease coding sequence, one promoter and a single RNA guide [26, 35].

3.2. CRISPR/Cas9 delivery and gene therapy

The CRISPR/Cas9 complex can be introduced into cultured cells and single-cell embryos in the form of DNA, RNA, or protein [36]. The DNA encoding Cas9 and gRNA can be delivered into the cell using plasmid and viral expression vectors. RNA or protein has been introduced through microinjection, liposome-mediated transfection, electroporation, and nucleofection.

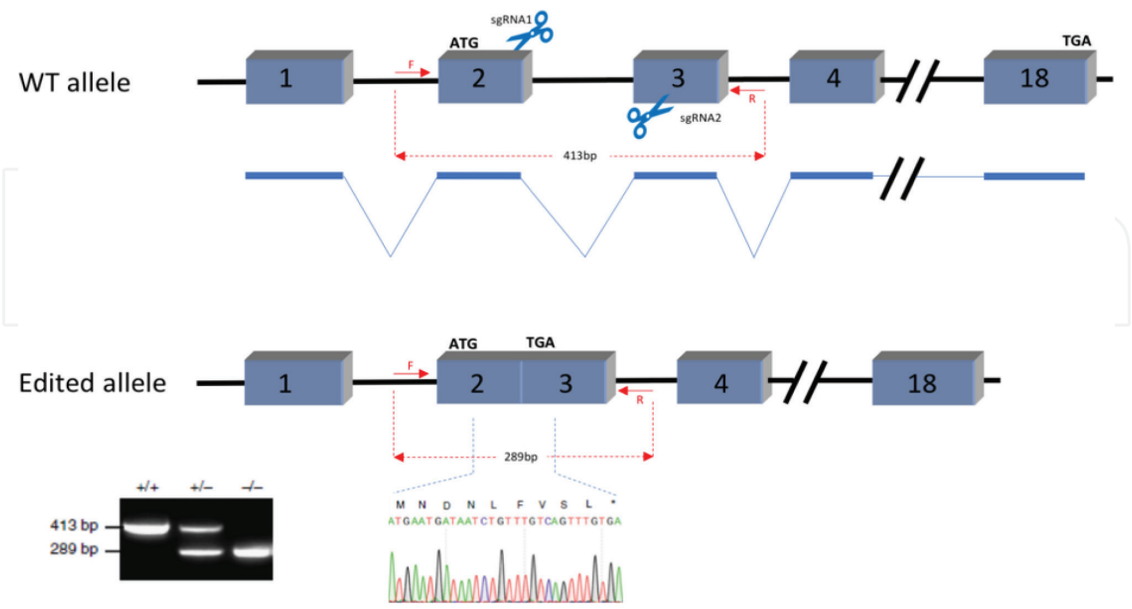

Figure 10. Schematic representation of Six6os1 WT and edited allele. Two sgRNAs were used to produce a deletion between exon 2 and 3. As a consequence, a premature stop codon appears at the beginning of exon 3. The edited allele can easily be detected by PCR [34].

However, the delivery formats of mRNA and protein pose certain technical challenges *in vivo* and viral-based *in vivo* genome editing remain a popular choice for achieving the 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 used 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 list the general characteristics and applications of various viral delivery vectors.

Currently, adenoviral vectors and γ-retroviruses are the most commonly 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 kb (ad) and 7 kb (rt/lt), allowing the accommodation of 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), insertional oncogenesis (rt/lt), generation of a replication-competent lentivirus (rt/lt), immunogenicity, and toxicity (ad) are risks that should be taken account in *in vivo* gene therapy.

In contrast, the AAV system has major advantages for research and therapeutics, including very low immune response and toxicity. AAVs remain in the cell as episome, avoiding insertional mutagenesis by random integration into the host genome. In fact, there are no human diseases related to them, and they can exist long term as concatemers in nondividing cells for stable transgene expression [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 to encode 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

Guide	Editing efficiency (%)	TIDE-predicted indels
Bcr-Abl sgRNA	85.0	+1 bp (17.5%), −1 bp (9.1%), −2 bp (4.8%) −3 bp (3.4%), −4 bp (6%), −6 bp (1.8%), −8 bp (18.9%), −11 bp (10.2%), −18 bp (5.1%)
TK-ABL 1 sgRNA	54.6	+1 bp (14.9%), −1 bp (8%) −2 bp (5.2%), −10 bp (17.6%)
TK-ABL 2 sgRNA	68.8	+1 bp (30.8%), −1 bp (5.9%), −2 bp (4.8%), −4 bp (15.2%), −14 bp (5.1%)
Mock sgRNA	0.0	

Table 2. Indels induced by each sgRNA predicted by the TIDE algorithm.

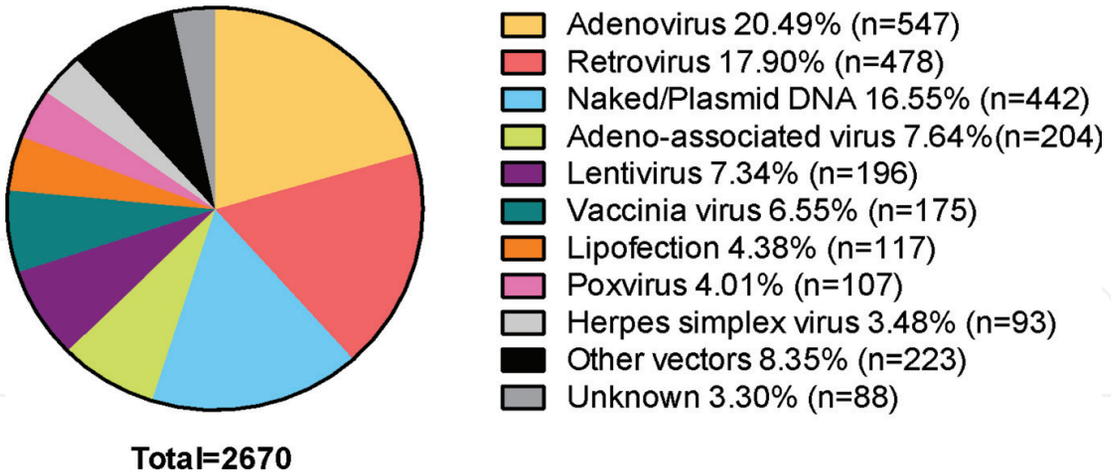

Figure 11. Delivery systems commonly used in gene therapy clinical trials.

Characteristics of 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 nondividing	Dividing or nondividing	Dividing or nondividing	Dividing or nondividing
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 frequency of host genome integration events)	No
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 *in vivo*, optogenetics	Recombinant proteins and vaccine production
Clinical trials	Very popular	Very popular	Popular	Increasingly popular	Growing interest

Table 3. Viral delivery systems most commonly used in gene therapy.

AAV vectors are able to express SaCas9, and one sgRNA has been described that appears to be 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 mouse liver. In this study, the authors observed modification in >40% genes, accompanied by significant reductions in serum Pcsk9 and total cholesterol levels [35].

Another problem 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 the search for AAV variants that can efficiently infect HSC or lymphoid cells has yet to identify any candidates [54].

All these advantages have led to increases in the number of studies using AAV vectors to deliver the CRISPR components in animals and in clinical trials for gene therapy.

3.3. CRISPR-Cas9 sgRNAs: "Superguides" for interfering with (onco)gene expression

When a cancer cell is the target, a delivery strategy that can result in the expression of Cas9 and an oncogen-specific sgRNA in all infected cells is desirable. This is especially critical for *in vitro* gene therapy where the expansion processes from a selected edited cell are 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 at knocking out the oncogene. In this way, for the vast majority of knockout studies where the edited cells or mice can be selected, the sgRNA targets different positions within the chosen exon, avoiding boundaries. In most of these cases, the designs follow off-target 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 a sgRNA targeting the exon boundary. Following this strategy, the generation of null alleles would be increased by two ways: probability of producing a frameshift mutation and 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, pioneering genetic studies indicated that many of the thalassemia mutations in the β-globin gene affect splice sites and give rise to aberrant splicing patterns [56, 57]. Recent studies have demonstrated that a splicing mutation in the STAR gene is a loss-of-function mutation that produces an aberrant protein [58]. Besides, non-sense-mediated mRNA decay (NMD), a conserved biological mechanism that degrades transcripts containing premature translation termination codons, could help secure 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]. Several mutations of splice donor sites that cause loss of gene function have recently been identified. A novel mutation at a splice donor site that was predicted to lead to skipping of exon 10 of the PLA2G6 gene was found in a homozygous state in infantile neuroaxonal dystrophy patients. This variant was correlated with very strong loss of function, providing further evidence of its pathogenicity [60]. Mutations in the

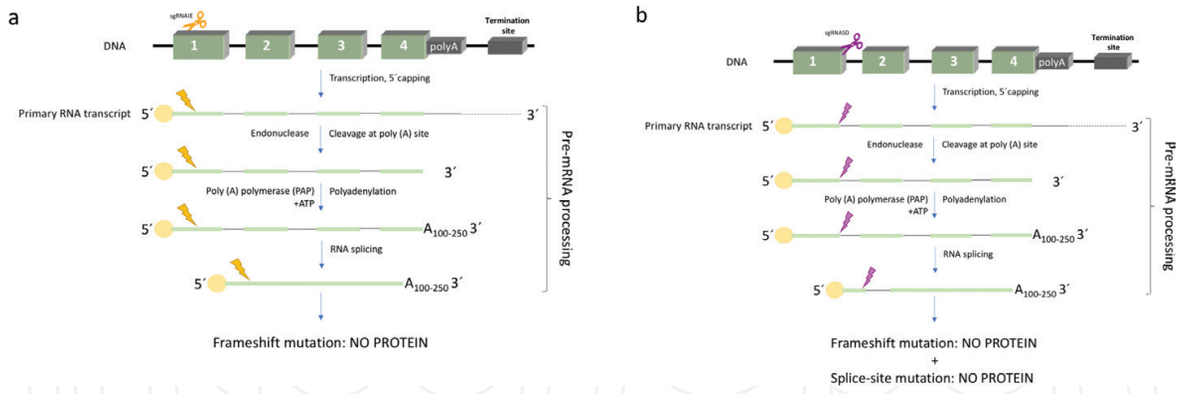

Figure 12. CRISPR/Cas9 design against sequences involved in intron processing.

ectodysplasin A1 gene (EDA-A1) at 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 the 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 function of the MCM9 gene, which is involved 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 give rise to premature stop codons. However, if DSBs are induced near the boundaries of the target exon, then the canonical splicing pathway could also be altered. In that case, to the probability of producing frameshift, mutations should be added that of interfering with canonical pre-mRNA splicing (**Figure 12B**). Even if the CRISPR/Cas9-induced mutation did not produce a frameshift mutation, at least this strategy would offer the possibility of producing nonfunctional oncogenes by splice-pathway alteration. It has recently been shown that CRISPR/Cas9-mediated alterations at 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].

An unpublished study has compared the efficiency of generating null alleles by CRISPR/Cas9 sgRNAs targeting exon boundaries. The authors compared the efficiency of producing null alleles inducing DSBs in a central position of the critical exon with DSBs close to the splice donor site on the exon. The study, which was carried out in a variety of genes, species and systems, revealed an increase in knockout efficiency using sgRNA guides targeting the splice-donor site of the chosen exon.

4. Conclusions

Genome-editing nucleases like the popular CRISPR/Cas9 enable knockout cell lines and null zygotes to be generated by inducing site-specific DSBs within a genome. In most cases, when a DNA template is not present, the DSB is repaired by nonhomologous end joining,

resulting in small nucleotide insertions or deletions that can be used to construct knock-out alleles. However, for several reasons, these mutations do not produce the desired null result in all cases, giving rise to a similar but functionally active protein. This undesirable effect could limit the therapeutic efficiency of gene therapy strategies that focus on abrogating oncogene expression by CRISPR/Cas9 and should be taken in account. The use of an 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.

Acknowledgements

The authors wish to express their sincere thanks to Dionisio Martín, Alberto Pendás (Spanish Research Council, CSIC), for their scientific assistance. We thank all the members at the Transgenic Facility (Nucleus, University of Salamanca), Verónica Alonso and Adrian Montaño for their technical assistance and contribution to our CRISPR/Cas9 studies.

Conflict of interest

The authors declare that they have no conflicts 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), a Novartis grant, and by the Junta de Castilla y León Fondos FEDER (SA085U16), and to the Programa Propio de Ayuda a la Investigación of the University of Salamanca.

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