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CRISPR: History and perspectives to the future

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ABSTRACT

This review summarizes the information about the history and future of the CRISPR/Cas9 method. Genome editing can be perceived as a group of technologies that allow scientists to change the DNA of an organism. These technologies involve the deletion, insertion, or modification of the genome at a specific site in a DNA sequence. Gene therapy in humans has a perspective to be used to eliminate the gene responsible for a particular genetic disorder. The review focuses on the key elements of this promising method and the possibility of its application in the treatment of cancer and genetic diseases.

1. Introduction of genome editing

Over the past two decades, a long-standing goal of researchers has been to develop cost-efficient and reliable ways to make precisely targeted alterations to the genome of living cells. Genome editing can play a vital role in solving problems in a broad spectrum of fields. Gene therapy in humans has a perspective to be used to eliminate the gene responsible for a particular genetic disorder. In agriculture, on the other hand, manipulating plant DNA could help enhance crop yields and control plant diseases (Synthego).

A new tool based on a bacterial clustered regularly interspaced short palindromic repeats - associated protein-9 nuclease (CRISPR/Cas9) from *Streptococcus pyogenes* has caused a big commotion in recent years [1].

2. History of gene engineering

CRISPR/Cas9 technology emerged in 2012. Since then, the techniques for targeted and precise manipulations of DNA sequences in living cells have played a crucial and dominant role in biology. Although CRISPR has become almost a synonym with gene editing, it is not a new concept and not nearly the first technology developed to edit DNA. The history of genome editing goes back to the 1970 s when researchers successfully employed transgenic mice [2]. It was, however, impossible to carry out a targeted insertion into the genome of a cell by this technique. The limitations have led scientists over the world to a united effort to develop different gene targeting technologies.

The first gene targeting system, introduced in 2005, was zinc finger nucleases (ZFNs). The recognition of the specific sequences of DNA and targeted insertion into the genome could be executed [3]. A few years later, in 2010, it was followed by Transcription activator-like effector nucleases (TALENs) [4].

3. The CRISPR/Cas9 technology

In 2012, it was discovered that the bacterium *Streptococcus pyogenes* could be adapted for gene engineering. This system consists of the "Clustered regulatory interspaced short palindromic repeat" CRISPR of RNA, which acts as a guide, and the Cas9 working as an endonuclease and enabling double-strand breaks (DSBs) [5].

The differences between CRISPR and two previously described genome editing systems are shown in Table 1. The critical difference in CRISPR compared to previous approaches is the use of a short RNA sequence serving as the specificity-determining element for DSBs. Moreover, CRISPR is simpler by requiring only the single guide RNA (sgRNA) and not the engineering of a site-specific nuclease that is time and money-consuming [6].

The CRISPR-mediated genome editing system is a powerful tool for genetic manipulation with various possible applications in biological sciences and clinical medicine. The system naturally evolved in

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Abbreviations

AAV	Adeno-associated virus
Acr	anti-CRISPR
AML	Acute myeloid cancer
Cas9	CRISPR-associated protein-9 nuclease
CRC	colorectal cancer
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
dCas9	dead Cas9
DDR	DNA-damage response
DSB	double-strand break
dsDNA	double-stranded DNA
gRNA	guide RNA
HDR	homology-directed repair
HSPCs	hematopoietic stem and progenitor cells
IBD	inflammatory bowel disease
MV	microvesicles
NHEJ	non-homologous end-joining
PAM	protospacer adjacent motif
PC	pancreatic cancer
PKC	protein kinase C
pre-crRNA	precursor CRISPR RNA
RCC	renal cancer cells
RNP	ribonucleoproteins
sgRNA	single guide RNA
SSBs	single-stranded breaks
ssDNA	single-stranded DNA
TALEN	transcription activator-like effector nuclease
Tra-crRNA	trans-activating CRISPR RNA
ZFN	zinc finger nuclease

Table 1
Comparison of genome engineering tools.

Properties	ZNFs	TALENs	CRISPR
DNA-binding moiety	Protein	Protein	RNA
Nuclease	*Fok*I	*Fok*I	Cas
Target recognition size	18–36 nucleotides	30–40 nucleotides	22 nucleotides
Toxicity	Variable to high	Low	Low
Ease of targeting multiple targets	Low	Low	High
Complexity of design	Very complex	Complex	Simple
Off-target effects	Moderate	Low	Variable

prokaryotes to protect against mobile genetic elements, in particular, bacteriophages and plasmids. In recent years, several engineered versions of CRISPR have been developed mainly due to th is technique's programmable nature and minimal requirements. Nowadays, CRISPR-based technologies enable efficient targeting and alteration of DNA in living cells from dozens of species (including humans and other eukaryotes) and are widely adopted by the scientific community. Precise editing can be utilized in personalized gene therapy to correct inherited monoclonal diseases or sequence-specific targeting of pathogens to treat infectious diseases, as well as for many other applications. However, the application of CRISPR simultaneously brings many practical and technological challenges mainly associated with delivery strategies, the control of repair pathways, off-target and on-target effects, and controversial ethical issues [7].

3.1. CRISPR system classification

CRISPR systems are classified into six types, which are further grouped into two classes based on the sequence and the structure of Cas proteins. While types IV-VI have only recently been identified, types I-III have already been thoroughly studied, and the type II CRISPR system is the most widely applied. Unlike the simplest type II, types I and III are not used to modify genes due to their complexity. The type I system contains Cas3 protein, which utilizes the DNase domain and helicase to degrade the target. Subtypes of type II system have Cas9 gene and genes for Cas1, Cas2, and Cas4 (in case of II-B subtype) and are used in gene therapy thanks to the simplicity and the multifunctional Cas9 protein. Various point mutations in Cas9 have been introduced to provide even higher specificity [8]. The last out of well-studied types, type III, contains gene encoding Cas10 protein [9]. This endonuclease has a unique mechanism with dual destruction of both RNA and DNA [10].

Novel types of RNA-guided Cas proteins are being searched to develop simple and particular CRISPR-based technologies. One of the main aims is to minimize the size of employed nucleases to make it easier to package their genes for delivery. One of the recently identified miniature CRISPR systems are employing is a family of highly compact effector proteins named Cas14, with the size of 400–700 amino acids. Cas14 proteins are RNA-guided nucleases targeting single-stranded DNA (ssDNA) by a single RuvC nuclease domain without the requirement of PAM. Despite their small size, Cas14s operate with larger RNA scaffolds [11]. The newest class 2 type VI system, CRISPR/Cas13a (also known as C2c2) from the bacterium *Leptotrichia shahii*, can recognize and cleave single-stranded RNA molecules, working as an RNA-guided ribonuclease [12]. The researches concerning Cas14 and Cas13a could be economically crucial for engineering the interference against plant ssDNA or RNA viruses [13,14]. The classification of CRISPR endonucleases is shown in Table 2.

3.2. CRISPR pathways and main components

3.2.1. Pathways of DSB repair

The enzymes used in the gene targeting methods can recognize required sequences on the genome, and subsequently introduce DSBs in the target nucleic acid sequence and permit a cell repair process to prevent lethality. The reparation process may be performed in two different ways, homology-directed repair (HDR) and non-homologous end-joining (NHEJ), both of which can be used to acquire the desired editing outcome.

- Homology directed repair

  HDR is one of the two major DNA pathways for DNA damage repair. It requires a homologous template for restoration which may be provided endogenously or exogenously. HDR may be used for adding or removing specific DNA sequences at the location of the DSBs. However, it is considered to be less efficient compared to NHEJ [15].
- Non-homologous end-joining

Table 2
Classification of CRISPR endonucleases.

Class	Type	Protein
1	I	Cas3
	III	Cas10
	IV	Scf1
2	II	Cas9, Cas1, Cas2, Cas4
	V	Cas12
	VI	Cas13

The second process, known as NHEJ, uses the cell repair machinery to re-ligate the ends of DNA breaks without a repair template. However, this repair process is error-prone and relatively imprecise, as there is a high chance of insertion or deletion mutations of DNA, which subsequently may result in disruption of translation of the targeted gene [16].

3.2.2. Components of CRISPR/Cas9 system

CRISPR/Cas9 method is a three-component system that consists of an endonuclease (Cas9) and two small RNAs –CRISPR RNA (crRNA), which is a sequence-specific targeting element, and trans-activating crRNA (tracrRNA) that links Cas9 with the crRNA [17].

The endonuclease Cas9 is a large multi-domain and multifunctional DNA endonuclease. Its primary purpose is to cut the genome at the desired location. While in the native CRISPR/Cas systems, various enzymes are required for endonuclease activity, only one CRISPR protein (Cas9 or its variant) is necessary for genome editing. This nuclease has all the necessary components for:

- binding to gRNA, this bind subsequently enables Cas9 to cut a particular genomic locus out of many possible loci
- binding to target DNA in the presence of a gRNA, provided that target is upstream (5′) of a PAM
- cleaving Target DNA, what results in a DSB [18]

Cas 9 protein includes two distinct lobes, the recognition (REC) lobe and the nuclease (NUC) lobe. The latter contains two endonuclease domains, a RuvC-like nuclease domain and an HNH like nuclease domain, crucial for the function of Cas9 in the last step. During the cleavage of the target DNA sequence, the RuvC and HNH-like nuclease domains cut both DNA strands, resulting in DSB 3 base pairs upstream of the PAM motif. The HNH-like domain is responsible for cleaving the complementary strand, while the RuvC-like domain cleaves the second – non-complementary strand [5,19]. Except for the two nuclease domains, Cas9 contains REC I, REC II, Bridge Helix and PAM-Interacting domain. Rec I domain is the largest one and responsible for binding sgRNA, bridge helix is vital for initiation of cleavage activity, and PAM-Interacting domain is crucial for PAM specificity [19,20] .

3.2.3. The single guide RNA (sgRNA) or guide RNA (gRNA)

Cas9 is led to the target location by two RNAs: the crRNA, recognizing and pairing with a sequence of 20 nucleotides within the targeted genome, thus defining the target for Cas9, and the tracrRNA, linking the crRNA to Cas9 and facilitating maturation of crRNAs from precrRNAs. In most CRISPR-mediated genome editing systems, these two RNAs (crRNA and tracrRNA) have been combined into one molecule called sgRNA or gRNA. The sgRNA contains a 20-nucleotide target sequence that refers Cas9 to a specific genomic locus and the sequence necessary for Cas9 binding [18]. Overview of different tools for the design of highly specific gRNA is summarized in this review [21].

3.2.4. Protospacer adjacent motif (PAM)

For binding of Cas9 endonuclease to the target genomic locus both, already described 20-nucleotides long complementary sequence of sgRNA and this 3-base pair sequence termed PAM, are required. PAM is a short sequence located on the target DNA strand, which is necessary for the endonuclease activity of Cas9. In the absence of this short sequence, even an entirely complementary sequence cannot be recognized; therefore, the requirement of PAM in the genome is considered one of the major restrictions of this technique [22]. The native PAM sequence for the *Streptococcus pyogenes* Cas9 is 5′-NGG-3′ (where G means guanine and N stands for one of the four DNA bases). However, more than 20 additional Cas9 homologs have been isolated from a variety of bacterial species. These homologs have different PAM sequences, and because they do not cross-react, it is possible to use multiple Cas9s simultaneously [18]. To cut the double-stranded DNA (dsDNA) by Cas9, a PAM sequence needs to be located immediately downstream of the 3′ end of the site targeted by the gRNA. This allows the cleavage of the genomic sequence by Cas9, generating DSB caused by two endonuclease domains, HNH and RuvC-like nuclease domains, occurring three nucleotides upstream of the PAM motif [17,23–25]. Cas9 generated DSB and schemes of gene disruption or precise gene editing are illustrated in Fig. 1.

3.3. CRISPR in genome editing

Up to now, three variants of the Cas9 endonuclease have been used in genome editing. The first, Cas9, can cleave dsDNA at a specific location, causing DSB and subsequently activating the repair mechanisms. DSBs are repaired by either the cellular NHEJ pathway which results in insertions or deletions that disrupt the addressed locus or the HDR pathway, if the donor template is provided (Fig. 2 A). This HDR pathway leads to precise substitution mutations [26]. Increased precision was achieved by Cong and colleagues [27], who developed a mutant form of Cas9, known as Cas9D10A. This mutant nuclease has only nickase activity (RuvC domain of Cas9 is inactivated), thus cleaving only one strand of DNA. Unlike the first variant of Cas9, Cas9D10A does not activate NHEJ (Fig. 2 B). When the repair template is supplied, the break is mended by the HDR pathway [28]. Nowadays, several modifications of nickases are suitable not only for DNA but also for RNA [29].

Newly prepared endonuclease Cas9D10A is suitable for minimizing off-target mutations, and it has a high level of on-target mutagenesis [30]. This endonuclease was successfully used to generate isogenic knockouts for genes: DNA-damage response (DDR), MDC1, 53BP1, RIF1, P53 and Lamin A/C (the nuclear architecture proteins) in three human cell lines [30].

The third variant is a nuclease-deactivated Cas9, referred to as 'dead' Cas9 (dCas9). This variant is created by mutations of nuclease domains,

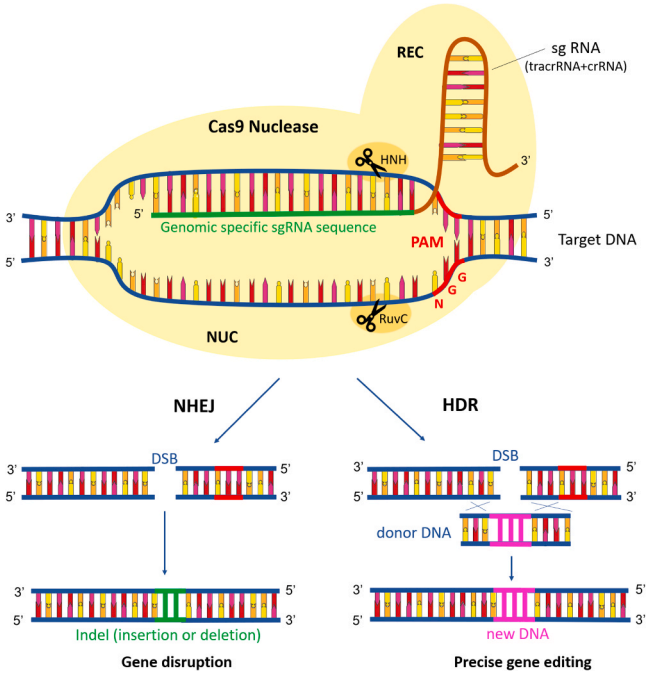

Fig. 1. Recognition lobe of Cas9 endonuclease is responsible for binding sgRNA, consisting of crRNA and tracrRNA, which based on sequence complementarity recognizes and binds to the target genomic sequence. The nuclease lobe is responsible for recognizing a short 2–5 base pair sequence PAM that immediately follows a 20-nucleotide sequence, and without which the targeting would be impossible. Each of the two nuclease domains, HNH and RuvC, nicks one of the DNA strands generating a DSB. Subsequently, DSB is repaired either by NHEJ, an error-prone pathway that may result in the creation of Indels that may disrupt the gene or HDR in the presence of a donor construct.

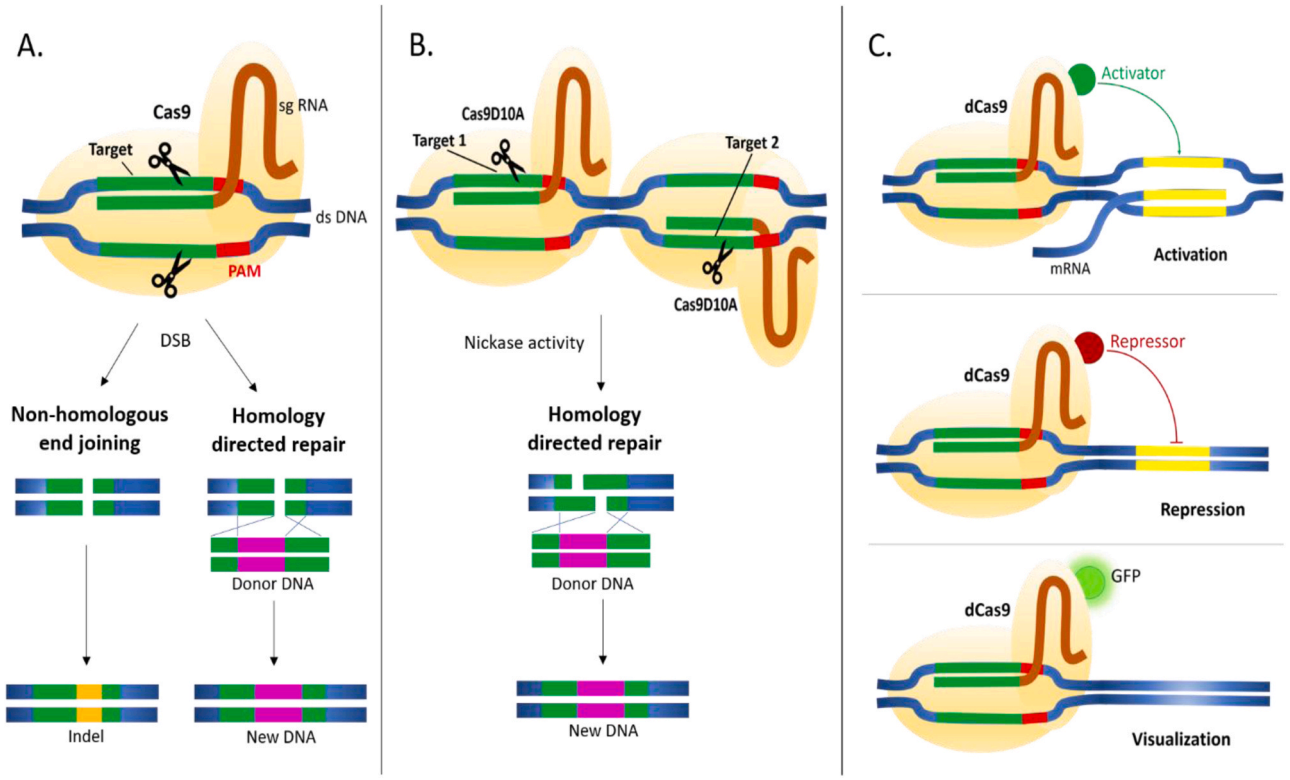

Fig. 2. Modified variants of different types of Cas9. A. Cas9 endonuclease cleaves dsDNA site specifically generating DSB with subsequent activation of the repair process. This can be either NHEJ that results in indels and disruption of the target sequence, or by providing donor DNA, precise mutations can be made via HDR. B. CAS9D10A – a mutant form of Cas9 with only nickase activity makes a site-specific single-strand nick. To introduce DSB, two sgRNA can be used. When donor DNA is supplied, DSB can be mended by HDR. C. Nuclease-deactivated Cas9 (dCas9) might be fused with different effector domains, for instance, transcriptional activators, repressors, or fluorescent proteins.

HNH and RuvC domain, resulting in inactivation of their cleavage activity. However, these mutations do not prevent DNA binding [5]. Therefore, the third variant can be used to precisely and specifically target any region within the genome without its cleavage. This variant is used for non-editing applications, for instance, visualization or purification of genomic loci [31]. This dCas9 is routinely utilized for epigenetic modification of regulatory regions of genes of interest (Fig. 2 C). The use of different types of this nuclease is summarized in these reviews [32,33]. Vojta [34] used this method for epigenetic modification of regulatory regions of human genes BACH2 and IL6ST. These genes are crucial regulators of autoimmune responses in humans. Their dysregulation could cause diseases such as inflammatory bowel disease (IBD) and lupus erythematosus. These dCas nucleases can be used for activation or repression of transcription, tracking cells prepared by fusion with different effector domains or base editing [35].

3.3.1. *Delivery methods*

The safe delivery method of the CRISPR-Cas9 editing tool is a critical factor for therapeutic safety and efficacy. The CRISPR system can be delivered:

- as a plasmid DNA containing genes for Cas9 and gRNA,
- by Cas9-sgRNA ribonucleoproteins (RNP),
- in mRNA of Cas9 and sgRNA form [6]

CRISPR delivery methods are presented in Fig. 3.

The crucial delivery criteria include minimal cytotoxicity, effective cell targeting and rapid elimination of CRISPR elements [36]. Among the most widely used delivery methods belong delivery vectors, out of which Adeno-associated virus (AAV) is the most common vector for in vivo delivery [37]. Compared to other viruses, AAV is less immunogenic and has a lower risk of carcinogenesis [38]. However, AAV also has some disadvantages:

- the inserted transgene might disrupt an important gene
- the virus has a limited packaging capacity (max. capacity 4.7 kb)

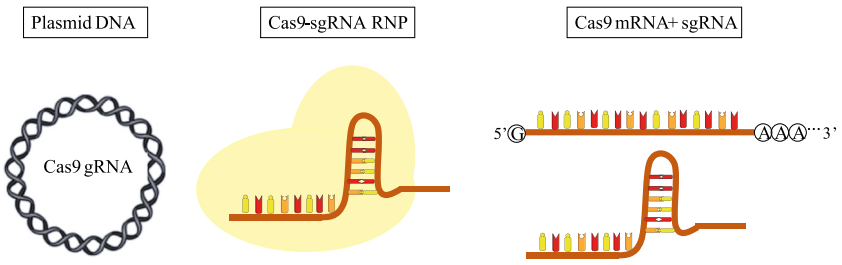

Fig. 3. Delivery methods of CRISPR-Cas9. A: plasmid DNA: the Cas9 and gRNA are encoded from the plasmid DNA, B: Cas9-sgRNA RNP: the complex is delivered via protein Cas9 and mRNA for sgRNA, C: Cas9 mRNA and sgRNA: these two components (as mRNA) form a complex suitable for delivery.

- the continuing presence of the delivery system might increase the chance of off-target effects

Despite the disadvantages, AAV is being extensively used for its high delivery efficiency and remains a crucial delivery vector in gene therapy [39].

Alternatively, Cas9-sgRNA ribonucleoproteins (RNP) could be used, owing to their rapid cell clearance and thereby minimizing the off-target effects [40].

To avert the risks of continuous expression, an ex vivo approach can be performed. By this approach, the target cells are genetically altered outside of the organism and then reintroduced back. The major advantage of this method is greater safety for patients. Still, it has challenges such as the retention of in vivo function of the cells outside of the organism and the expansion in culture. Besides, this method can be applied only for certain types of cells, such as hematopoietic stem and progenitor cells (HSPCs) and T cells that can survive in the culture [41]. Although ex vivo therapy is successfully applied for hematological diseases and cancer immunotherapy, tissue-specific disorders cannot be targeted in this way. To target them, CRISPR components need to be delivered by intravenous or local injections. After delivering to the circulatory system, the targeted expression of CRISPR components can be modulated through tissue-specific promoters for providing at specific organs [42].

A new delivery method for CRISPR-Cas that uses adopted epithelial cell-derived microvesicles (MV) as a carrier of CRISPR components to the cancer cells was recently developed [43].

Among the nonviral methods of delivery belong electroporation, nucleofection, and microinjection. Although these methods are averting virus-associated risk, other drawbacks are noticed. For instance, microinjection can be challenging to perform, and it is used only for ex vivo. Similarly, electroporation is also used primarily for ex vivo delivery. Still, it can be applied in vivo for specific tissues, however, with the risk of permanent permeabilization of treated cells [44].

Nonviral in *vivo* delivery such as nanoparticles enables control throughout dosage, reduction in nuclease expression, and subsequently the minimization of off-target cleavages [45]. Although the nanoparticles enable this timing control, it has some severe disadvantages:

- the process of packaging into small particles can be challenging
- its biological activity needs to be maintained until the nucleus is reached
- the nanoparticle materials should be non-immunogenic and biocompatible [36].

To summarise this knowledge, we can say that the CRISPR/Cas9 system has advantages such as simplicity and versatility, while other genome-editing technologies were not proved to have these characteristics. The clinical application of these technologies is dependent on the effective delivery of genome editing components to the target cells. The nanoparticle delivery system as polymer-based, lipid-based, and rigid inorganic nanoparticles [46] have been designed for the CRISPR/Cas9 system. These innovations make nonviral vectors for CRISPR/Cas9 delivery very promising for the future in the clinical application. Cas9 protein/sgRNA direct delivery shows lower off-target impact, high efficiency, and rapid action [47]. Fig. 4 summarizes all the delivery methods we described above.

3.4. CRISPR in cancer treatment

Since discovering its gene-editing capability, many tumor models with a knockout of tumor-suppressor genes and point mutations were generated for research purposes.

In many cases, cancer is the result of:

- genetic mutations,
- aberrant expression of specific genes,

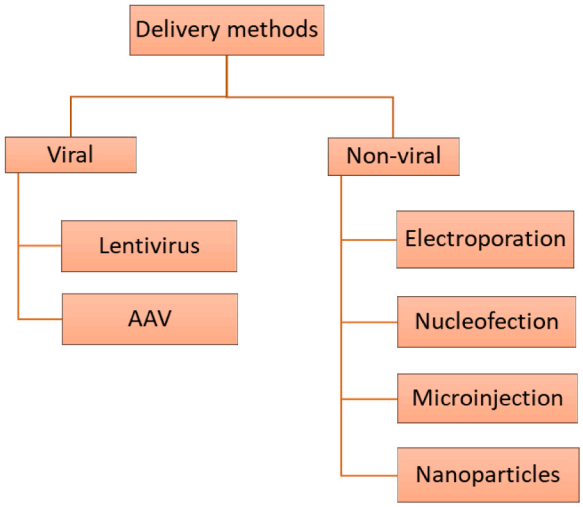

Fig. 4. Scheme of delivery methods of Crispr Cas9 components to target cells: the main two parts are viral (lentivirus or AAV) and nonviral methods (electroporation, nucleofection, microinjection or nanoparticles).

- epigenetic changes.

Therefore, CRISPR-Cas9 gene editing is a promising tool for curing cancer.

3.4.1. CRISPR genome, epigenome, transcriptome editing

Several essential genes in cancer cells have a very high genetic and epigenetic aberration in their regulatory regions. Epigenetic modifications, such as DNA methylations, are reversible. Although, the therapy with demethylation agents, has been used in the past to treat cancer, it has not achieved as high specificity of epigenetic editing as CRISPR/dCas9 [34]. Therefore, a CRISPR-Cas9 based approach of aberration correction might be a promising therapy. Moreover, this technology can be applied for the regulation of cancer epigenome [33].

The inhibition or activation of tumor-related genes was performed in the past with ZFs and TALEs equipped with transcriptional activators or repressor molecules. However, with the advent of dCas9, these transcriptional molecules were fused with dCas9 for more potent transcriptional activators such as VPR (VP64, p65, Rta) [48].

These tools were also used to reactivate hypermethylated tumor-suppressor genes in gastric, breast and lung cancer [49], and CRISPR epigenome editing was also used to repress factors that are involved in tumor-promoting inflammation. However, the epi/genetic editing has to face challenges as the tumors are highly heterogenic. In addition, genomic aberrations are different in tumor during various cancer stages and at other locations within a patient [50].

The progressive therapeutic strategy for cancer treatment is *ex vivo* modification of the patient's cancer cells via the CRISPR technique. Reinshagen et al. [51] used this approach for preparing cells susceptible for antitumor therapy. Then, these cells were administered back to the patient's body because of their ability to kill residual or metastatic cancer cells after primary treatment.

In colon cancer cell lines, protein kinase C (PKC) is usually impaired, and for instance, CRISPR-Cas9 mediated correction of mutation resulted in tumor growth reduction in a xenograft model [42]. Furthermore, CRISPR-based HDR was reported to correct oncogenic mutations in APC and ALK-F1174L in colon cancer cells and neuroblastoma, respectively [43].

Knock-in an HSV1-tk suicide gene via Cas9 reduced tumor size and cell death in human prostate and liver cancer models [30].

A very promising and effective treatment of cancer is Chimeric antigen receptor (CAR) T-cell therapy. The principle of this method is to isolate T-cells from blood and their *ex vivo* genetic modification via

CRISPR to express CARs specific for a patient's tumor. Subsequently, the modified T-cells are propagated and finally injected back to the patients [52].

The exosomes were successfully used to deliver CRISPR/Cas9-mediated *PARP-1* gene disruption into the ovarian cancer cell line SKOV3. Furthermore, this inhibition caused increased sensitivity of ovarian cancer cells to cisplatin. According to these results, we can conclude that exosomes might be a promising tool for cancer treatment in the future [53]. Fig. 5 shows nanoparticle delivery in vivo and their impact on genome modification.

Therefore, based on the reviewed studies, it can be expected that CRISPR-Cas9 technique can efficiently find new drug targets and genes responsible for chemotherapy resistance.

In addition to exosomes, novel nanoparticles were discovered for transportation of CRISPR/Cas9 components, through all the cell membranes, into the nucleus [54]. The main advantages of nanoparticles are non-immunogenic, low toxicity and targeted delivery [55].

3.4.2. *Targeting carcinogenic viruses*

Considering the initial role of CRISPR in antiviral activity as a part of adaptive bacterial immunity, it can be utilized for directly targeting and eliminating oncogenic viruses such as human papillomavirus (HPV) in cervical cancer, hepatitis B virus (HBV) and hepatitis C virus (HCV) in liver cancer, and Epstein-Barr virus (EBV) in nasopharyngeal carcinoma, Hodgkin's lymphoma and Burkitt's lymphoma. For example, in HPV, E6 and E7 genes and their promoters are responsible for malignant transformation; therefore a CRISPR-Cas9 based E7/6 inhibition leads to the inhibition of cervical cancer growth. However, although the above-mentioned is a promising strategy, it is a challenge to select a unique target as E6/7 genes are very short [36]. Moreover, the high variability of viral targets and targeting multiple viral loci simultaneously appear to be similarly challenging.

3.4.3. *Oncolytic virotherapy*

The CRISPR-Cas9 system can be applied to genetically modify certain viruses to enable them to replicate within the host and specifically infect and kill cancer cells inducing those responsible for an anticancer immune response [36]. Recently, Food and drug administration (FDA) approved the talimogene laherparepvec (T-VEC) virus for oncolytic virotherapy in advanced melanoma. This virus can specifically target cancer cells, initiate the production of granulocyte-macrophage colony-stimulating factor (GM-CSF), and increase anticancer immunity. Moreover, oncolytic viruses can be administrated with conventional chemotherapy and immune checkpoint inhibitors (e.g. anti-*PD-1*) [36]. Fig. 6 shows the possibilities of using the CRISPR method in the treatment of cancer.

Table 3 lists some of the successful applications of the CRISPR/Cas system in cancer research.

3.5. *Practical and technological issues in genome editing*

The CRISPR/Cas technology is a simple yet powerful and currently the most reliable tool for editing genomes of various organisms. However, CRISPR is prone to errors (off-target effects) and unintended outcomes (on-target effects); and demands improvements of several aspects, such as the efficiency of HDR and its safety in clinical usage.

Until this day, the CRISPR/Cas9 technique is successfully used for the treatment of genetic diseases as muscular dystrophy (Duchenne's syndrome), Cystic fibrosis, Wolfram syndrome, Leber congenital amaurosis, ß-Thalassemia, Sickle-cell disease, Huntington's disease, HIV and others [55–57].

Finally, yet importantly, this new technology faces ethical severe issues, mainly concerning human genetic engineering (with emphasis on germline editing).

3.5.1. *Off-target and on-target effects*

Specificity is essential for all targetable nucleases, mainly when applied in human therapy or food sources. In many instances, the RNA-guided nucleases can induce mutations at sites that differ from the intended target region. The tolerance of mismatches between the spacer

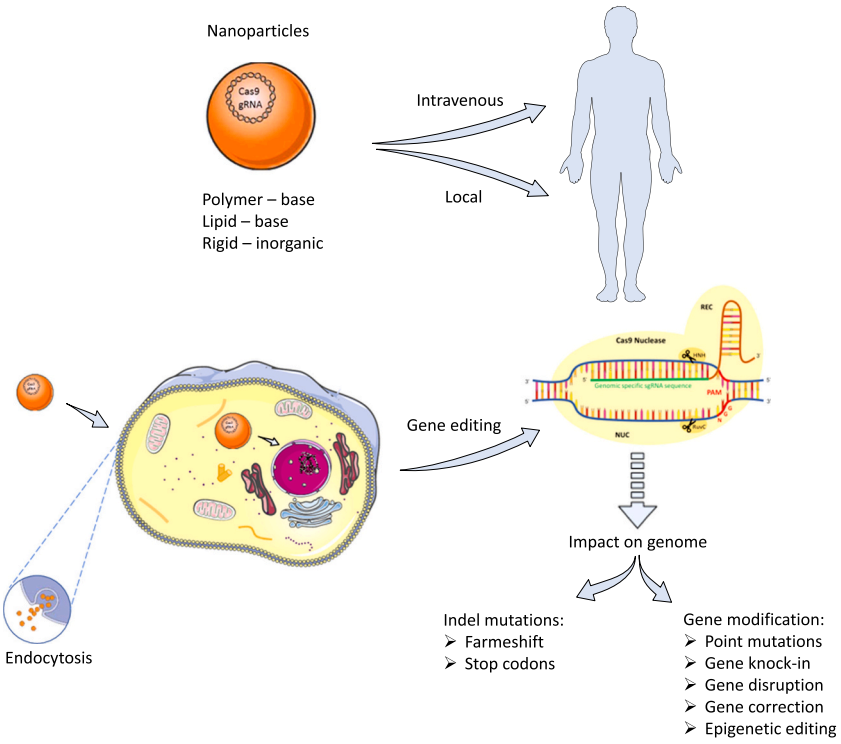

Fig. 5. Nanoparticles delivery in vivo and their impact on genome modification. Different types of nanoparticles could be delivered intravenously or locally to the patient body. Subsequently, nanoparticles travel through the cells into the nucleus, where genes could be modified.

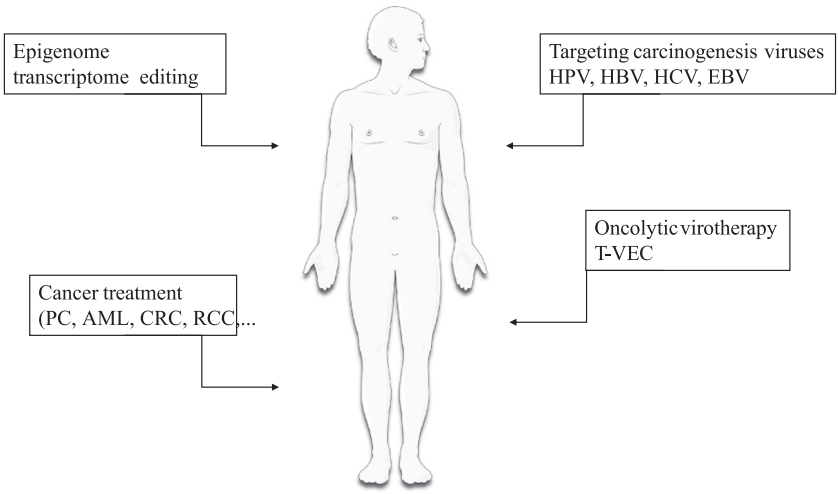

Fig. 6. The possibilities of using CRISPR method in the treatment of cancer: The CRISPR method could be used for: epigenome transcriptome editing, targeting of carcinogenic viruses, direct cancer treatment and oncolytic virotherapy.

Table 3
Use of CRISPR/Cas system in cancer research [74].

Cancer type	CRISPR/Cas9 delivery methods	Results	Reference
Pancreatic cancer	Multiplexed delivery into mice based on transfection	Editing of multiple sets of genes in pancreatic cells of mice	[75]
Acute myeloid cancer (AML)	Delivery into Hematopoietic stem cells by lentiviral vector	Loss of function of nine targeted genes analogous to AML	[76]
Breast cancer	Plasmid transfection into mouse cell line JygMC	Cripto-1, the stem cell marker, was shown to be a breast target	[77]
Liver cancer	Hydrodynamic injection into wild type mice	Mutation in the genes Pten and p53, leading to liver cancer in mice	[78]
Pancreatic cancer	Lentiviral/Adenoviral based delivery into somatic pancreatic mice cells	Knockout of gene Lkb1	[79]
Lung cancer	Plasmid transfection into HEK 293 – a human cell line	Chromosomal rearrangement among EML4 and ALK genes	[80]
Lung cancer	Lentivirus/Adenovirus mediated delivery	KRS gain of function and loss of function of Lkb1 and p53	[80]
Colon cancer	Plasmid transfection into DLD1 and human cell line – HCT-116	Loss of function in subgroups of protein kinase c	[81]
Colorectal cancer	Electroporation into organoids intestinal epithelium of human	Loss of function and directed mutation in genes APC, SMAD4, TP 53 and KRAS	[82]
Renal cancer	Mouse cell line Renca	TSG VHL knockout to induce cancer	[83]

and its protospacer during SpyCas9-mediated cleavage was observed already in the first papers concerning the application of CRISPR in genome editing [5,27,58]. The most crucial and least tolerant part of the sgRNA sequence is located at the 3′ end of the protospacer (sometimes called a "seed" region). On the other side, in the 5′ terminal region of the protospacer, up to six contiguous mismatches were noticed to be tolerated [5]. To lower off-target cleavage frequency while preserving on-target activity, several technologies have been developed. One of the approaches is a paired nicking by a Cas9 nickase mutant which creates two single-stranded breaks (SSBs) in such proximity to each other that it generates DSB [59]. Good efficiency is also shown using truncated sgRNAs with shorter regions of target complementarity than 20 nucleotides in length. Moreover, truncated sgRNAs coupled with paired nickases can further reduce off-target activity [58]. Alternatively, the focus can be directed to increasing the Cas9 specificity through rational structure-guided design. It was demonstrated that the neutralization of a positively charged groove HNH, RuvC and PAM interacting domains in enhanced SpyCas9 (eSpyCas9) could decrease off-target activity. The eSpyCas9 enzyme engineered in this manner has reduced the helicase activity [60]. Another engineered variant, Sniper-Cas9, was evolved in *E. coli* while maintaining the high on-target activity in comparison to the wild-type SpyCas9 [61]. On the other hand, the wild-type Cas9 ortholog from *Neisseria meningitidis* 33 (NmeCas9) has shown naturally high-fidelity editing capabilities with a nearly complete absence of unintended off-targeting. NmeCas9 is a very compact protein with only 1082 amino acids, making it better-suited for the size-restricted viral delivery [62]. Unwanted on-target effects involve mainly undesired outcomes coming from DSBs introduced by Cas9. It was shown that DSBs might result in unexpectedly long deletions causing more complex genomic rearrangements at the targeted site and potentially leading to pathogenic consequences [63]. In addition, the sensitivity of some cell types causes the induction of a p53-mediated DNA damage response and cell cycle arrest [11].

3.5.2. DSB repairs

To obtain precise genome editing mainly in potential therapeutic applications, employing error-free HDR-driven alternations is more favorable. Exogenous donor templates can be used to introduce point mutations or recombination sites or even introduce a whole gene of interest into a given locus. However, in mammalian somatic cells, HDR is limited by the more preferred but error-prone NHEJ at all cell cycle stages, and HDR is utilized in the S phase primarily [64]. To enhance genome editing by HDR, several approaches have been developed. For instance, the inactivation of one of the NHEJ pathway components (e.g., DNA Ligase IV) can cause the global inhibition of NHEJ [65]. However, NHEJ is important in genome maintenance, and global NHEJ inhibition strategies have a severe clinical impact, including growth delay and immunodeficiency [66]. Another approach in development is to fuse Cas9 to a dominant-negative mutant of tumor suppressor p53-binding protein 1 (DN-53BP1), a vital regulator of choice between NHEJ and HDR. The resulting Cas9–53BP1 fusion proteins significantly block the NHEJ activity by suppressing the recruitment of downstream NHEJ proteins at sites of DNA damage while upregulating the accumulation of HDR proteins [67]. Enhancing the probability of HDR can be induced by increasing the concentration of the donor DNA near the DSB site. This

can be achieved by directly conjugating sgRNA with donor DNA into one molecule through chemical modification at their terminal positions [68].

3.5.3. Other issues and observations

The efficiency of cleavage by CRISPR/Cas systems varies considerably in different genomic targets, even within the same type. One of the reasons could be histone dynamics in Cas9 inhibition by nucleosomes [69]. Cas effector proteins do not encounter nucleosomes within prokaryotes at their natural settings, and therefore, there is no selection pressure to acquire the capability of interacting with them. The failure of Cas9 to cleave at nucleosome-bound targets can be partly solved by prolonging the experimental time frame, as it is assumed that Cas proteins are fast enough to find the target site at the moment when there is no chromatin, e.g., during replication or active transcription. Nucleosomes interfere directly with Cas9 cleavage but do not affect zinc-finger nucleases [70]. Viruses evolved the ways to avoid the host's protective systems, including "anti-CRISPR" (Acr) systems which work as natural inhibitors. The significant observations that brought attention to anti-CRISPRs were phages not being cleaved by the host, and small proteins encoded within or near the CRISPR loci and the presence of self-targeting CRISPR arrays. Acrs can be used as the off-switchers for CRISPR/Cas systems to spatially, temporally and conditionally limit the Cas effector protein activity. Using a bioinformatic and experimental screening approach for identification, several inhibiting Acr proteins of NmeCas9 and Cas12a enzymes were detected. These proteins interact directly with Cas effector proteins and can be highly species-selective [71,72]. Another nascent challenge is the possible immune reaction activated in humans, as commonly used SpyCas9 and SauCas9 proteins originate in the infection-causing bacteria. The presence of anti-Cas9 antibodies was determined in 79% of donors staining against SauCas9 and 65% of donors against SpyCas9 [73].

4. Conclusion

This review summarizes current knowledge about CRISPR/Cas technology and its history from discovery until current understanding. We sum up some of the available variants of the endonuclease Cas9, the possibilities of delivery of individual components, and their use in specific genetic modification procedures. This progressive method is used not only in the modification of genes, but it can be very successfully applied in the treatment of genetic disorders and the treatment of various types of cancer. Although new drugs are being developed to treat the mentioned diseases, CRISPR/Cas technology has, from our point of view, much greater therapeutic potential. Only a thorough study of the molecular mechanisms of this method and all its components will allow its effective therapeutic use. We expect that such therapy could be the basis for personalized medicine in the near future.

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CRediT authorship contribution statement

Z. K: writing, editing and review of the manuscript and preparation of schemes, S. R: writing of the manuscript, preparation of schemes and language correction, P. M: writing of the manuscript and preparation of schemes, S. Dz writing of the manuscript, L.D: review of the manuscript and preparation of schemes.

Conflict of interest statement

The authors declare no conflict of interest.

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