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Current CRISPR-Cas gene editing systems and therapeutic

Abstract

In 2023 Casgevy (Exagamglogene autotemcel) became the first CRISPR-Cas therapeutic to be approved in the United States. This therapeutic is used to treat sickle cell anemia and represents the culmination of research in the field of CRISPR-Cas systems as a human therapeutic. As of 2022, there are around 71 clinical trials related to CRISPR in various stages. Unfortunately, CRISPR-Cas therapeutics can be expensive, as Cagevy is reported to cost 2.2 million USD per treatment. Fortunately, there is a good possibility the cost of CRISPR therapeutics can be reduced, as researchers today have more tools at their disposal to create gene edited products. These tools include more options at the Cas effector level, such as Cas9, Cas 12, Cas13, and xCas9. In addition, increased efficiency for CRISPR-Cas editing can be improved using guide RNA spacer modifications and RNA aptamers to improve Cas binding to the guide RNAs. Software such as CHOPCHOP and Synthego for guide RNA designs can help researchers visualize and improve guide RNA strategies. This paper will discuss current CRISPR-Cas systems and strategies used to make gene editing more efficient and provide a glimpse into the future of gene editing treatments by discussing current CRISPR-Cas therapeutics in clinical trials.

Keywords: Base editing, casgevy, Cas12, Cas13, CRISPR-Cas9, gRNA, PAM, prime editing, tracrRNA

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Abbreviations: CAGR, compound annual growth rate; CBE, cytosine base editor; cgRNA, circular guide RNA; crRNA, CRISPR RNA; CRISPR-Cas9, clustered regularly interspaced short palindromic repeats- CRISPR associated protein; dRNA, dead sgRNA; gRNA, guide ribonucleic acid; PAM, protospacer adjacent motif; pegRNA, prime editing guide RNA; sgRNA, single guide RNA; SpCas9, *streptococcus* pyogenes Cas9; T_m, melting point; tracrRNA, transactivating CRISPR RNA; USD, United States Dollar

Introduction

In 2020, Jennifer Doudna and Emmanuelle Charpentier won the Nobel Prize in Chemistry for their work on CRISPR-Cas9.[1] This achievement is the culmination of discoveries that date back to 1987 when scientists started studying DNA sequences that were described as clustered regularly interspaced short palindromic repeats (CRISPR).[2] Researchers eventually figured out how these CRISPR systems used RNAs to guide Cas proteins to cut DNA or RNA from viruses.[3] These systems subsequently revealed that programmable RNAs could help edit DNA sequences of interest.[4] Today, the CRISPR-Cas technology is estimated to be around 1.85 billion USD in market share.[5] This technology is ubiquitous, as we can find it in our food supply. These include tomatoes with higher nutritional value[6] and faster growing fish, such as red sea breams.[7] Treatment for sickle cell anemia through CRISPR has now been approved by the FDA.[8] Unfortunately, CRISPR-Cas 9 systems in general can have a failure rate of up to approximately 15%.[9] CRISPR-Cas systems such as prime editing and base editing can have low editing efficiency percentages between 4% to 17%[10] and 35% to 50%[11] respectively. To address some of these CRISPR editing deficiencies, we will discuss strategies such as spacer, scaffold, and chemical modifications of the CRISPR-Cas systems.

Market size of CRISPR and cas products

An article by Biospace estimated that CRISPR-Cas products in 2023 had a 1.85 billion USD market share in the United States.[5] Furthermore, the same article estimated that this CRISPR market share would rise to 8.59 billion USD in 2033. Another article by Precedence had a more bullish outlook, with an estimated market share of 16.55 billion USD by 2034 for CRISPR treatments (Figure 1).[12] The compound annual growth rate (CAGR) of CRISPR-Cas products predicted by Grand View Research is 16.5% from 2024 to 2030.[13] The report mentions CRISPR-Cas biomedical applications in the treatment of chronic illnesses accounting for 92% of the total segment in this area. Agriculture was also identified in the report as the fastest CAGR for CRISPR products between 2024 to 2030. Many agricultural products have had CRISPR-Cas gene modifications including wheat, maize, rice, millet, sorghum, bananas, soybean, barley, and many others.[14] CRISPR technology has also produced gene knock out and genetically modified mice by introducing double strand breaks in one cell stage embryos, reducing the time of creating genetically modified mice from one year to four weeks.[15] The highlight of CRISPR-Cas made products was the approval of Casgevy, formerly known as CTX001, a product where a hematopoietic stem cell CD34+ is genetically edited at the enhancer region of the BCL11A gene in order to treat sickle cell anemia.[8] Casgevy allows the increased expression of fetal hemoglobin to compensate for the loss of adult hemoglobin.[16] Casgevy is the first FDA approved CRISPR-Cas genome edited therapy.[8]

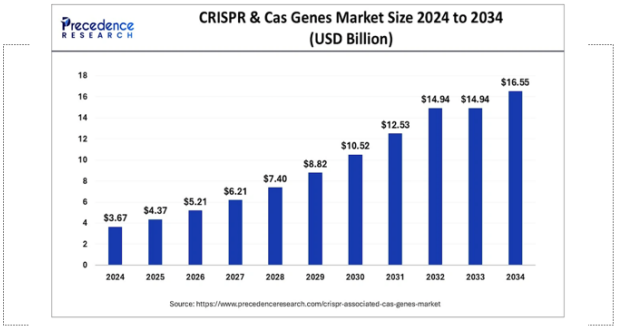

Figure 1 Bar graph illustrating the yearly growth of the CRISPR-Cas market from 2024 to 2034.[2]

This graph shows the growth of the CRISPR-Cas market from 2024 to 2034.

Basics of CRISPR-cas systems

The CRISPR-Cas 9 complex is composed of three components, Cas-9 endonuclease, guide RNA, and the PAM (protospacer adjacent motif).[17] The guide RNA consists of a tracrRNA (trans-activating CRISPR RNA) that acts as a scaffold for the Cas9 endonuclease and the crRNA (CRISPR RNA) has the series of nucleotides for hybridization of the gene section of interest. The Cas9 endonuclease has two nuclease domains, an RUVC-like section towards the N-terminal and a HNH like domain near the center.[17] The Cas9 makes a conformational change once bound to the guide RNA and cuts the nucleotides before the PAM, within the section of interest. The PAM is usually a three base sequence downstream from the CRISPR-Cas9 complex, depending on the effector used, and is necessary for this unit to operate (Figure 2). If we take a closer look at the sgRNA for SpCas9 (Streptococcus pyogenes Cas9), we can see that it is made up of six parts in addition to the crRNA and tracrRNA.[18] These parts include the spacer, lower stem, upper stem, bulge, nexus, and hairpins. The most important part of the sgRNA is the lower stem that is made up of complimentary bases, repeat region of the crRNA, and anti-repeat areas of the tracrRNA.[19] Without the lower stem, cleavage of the targeted site does not occur.[19] The hairpins help stabilize the sgRNA and Cas9 protein complex, whereas the nexus interacts with Cas9 and the target DNA for the DNA to be cleaved (Figure 3).[20]

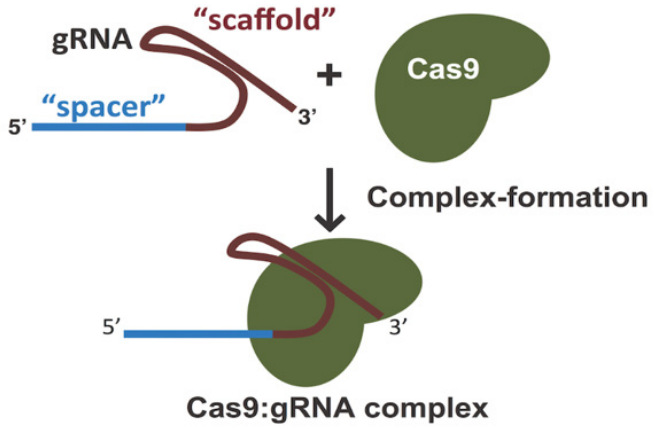

Figure 2 Figure showing the basics of a CRISPR-Cas9 complex.[5]

Diagram showing the components of the CRISPR-Cas9 complex.

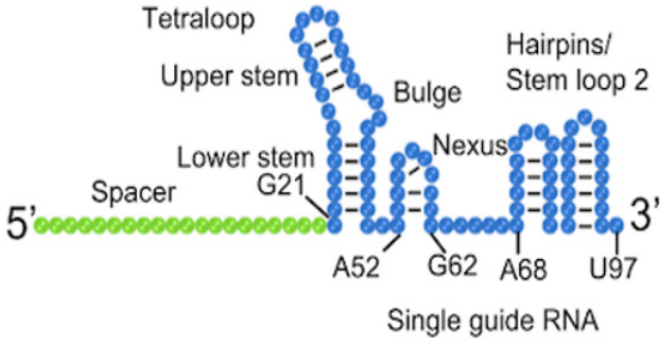

Figure 3 Diagram of a sgRNA and its components.[8]

This diagram shows the different parts of the sgRNA which include the lower stem, upper stem, tetraloop, bulge, nexus, and hairpins.

Current types of CRISPR-cas systems

CRISPR systems are named according to their CRISPR associated (CAS) effectors.[21] Class one CRISPR uses multiple effectors whereas class two uses one effector. Because of its simple architecture and single effector, class two CRISPR-Cas systems are the most popular with SpCas9, from *Streptococcus pyogenes*, effector being the most widely used.[20] Other Cas9 orthologs have more specific PAM sequences that allow increased specificity.[22] Examples of these systems include: SaCas9 from *Staphylococcus aureus*, that recognizes the PAM sequence 5'-NNGRRT-3', NmCas9 from *Neisseria meningitidis*, that recognizes the PAM sequence 5'-NNNNGATT-3', and CjCas9 from *Campylobacter jejuni*, with its recognized sequence at 5'-NNNNACAC-3'.

Another class two CRISPR system is Cpf1 (Cas12a) and includes *Francisellanovicida* (FnCpf1), *Acidaminococcus sp*. (AsCpf1), and *Lachnospiraceae bacterium* (LbCpf1).[20] These systems are directed to T rich PAM sites and create staggered cuts with 5' overhangs, which are beneficial for gene insertions in non-dividing cells.[23] Cas12a are able to create DNA cuts that are sticky due to the use of only the RuvC domain.[24] An advantage Cas12a has over Cas9 is the smaller size of the endonuclease due to a lack of tracrRNA, which allows it to be delivered by small capacity vectors such as adeno-associated viral vectors and lower cost of production.[17]

Cas13 is an RNA targeting nuclease.[25] There are four subtypes which include Cas13a, Cas13b, Cas13c, and Cas3d.[25] This system binds the guide RNA and can be used to do RNA knockdown, RNA editing, RNA imaging, RNA Splicing and regulation.[20] Both Cas12 and Cas13 can be used in mammalian cells.[20] Other Cas9 variants and their PAM sites can be seen in Figure 4.

Species/Variant of Cas9	PAM Sequence
Streptococcus pyogenes (SP); Sp-Cas9	3' NGG
SpCas9 D1135E variant	3' NGG (reduced NAG binding)
SpCas9 VRER variant	3' NGCG
SpCas9 EQR variant	3' NGAG
SpCas9 VQR variant	3' NGAN or NGNG
xCas9	3' NG, GAA, or GAT
SpCas9-NG	3' NG
Staphylococcus aureus (SA); Sa-Cas9	3' NNGRRT or NNGRR(N)
Acidaminococcus sp. (AsCpf1) and *Lachnospiraceae bacterium* (Lb-Cpf1)	5' TTTV
AsCpf1 RR variant	5' TYCV
LbCpf1 RR variant	5' TYCV
AsCpf1 RVR variant	5' TATV
Campylobacter jejuni (CJ)	3' NNNNRYAC
Neisseria meningitidis (NM)	3' NNNNGATT
Streptococcus thermophilus (ST)	3' NNAGAAW
Treponema denticola (TD)	3' NAAAAC

Figure 4 This table shows Cas enzymes and their corresponding PAM sites.[17]

This table shows the Cas variations and their corresponding PAM sites. It illustrates the variety of Cas enzymes that can be used depending on its location close to one of these PAM sites.

Applications Involving CRISPR cas systems

Gene knock in

Guide RNAs can be made to target a specific DNA sequence to be cleaved and then have a replacement DNA sequence to be inserted through either homologous or non-homologous directed repair.[26] For homologous directed repair knock ins, the donor DNA can be delivered through the use of plasmids, or as a single stranded oligonucleotide.[27] Homologous directed repair knock ins are more efficient when done

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during the G2/M cell cycle.[28] The insertion of Cas9 and gRNA into a cell can be done through the use of an expression vector or transfected directly as Cas9 mRNA and gRNA.[29] Gene knock ins with CRISPR-Cas can also be attempted with non-homologous end joining repair.[30] However this method tends to be error prone with many insertions and deletions at the repair junctions.[30]

Gene knock out

A guide RNA can target a gene of interest and cleave that area which will result in non-homologous end joining.[31] An example of this is using two CRISPR-Cas nikase mutants at opposite strands close to your target gene to create a double strand break in the DNA.[32] This will result in a NHEJ repair but may lower off target effects (Figure 5).[32]

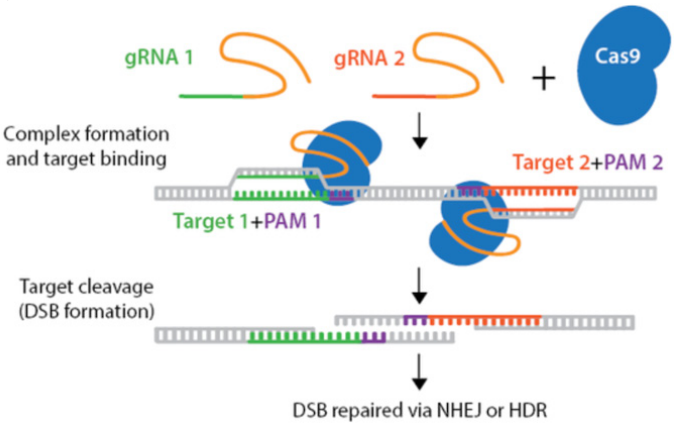

Figure 5 This image demonstrates mutant CRISPR-Cas nikases and their ability to introduce a double strand break for a knock out procedure.[32]

Technique using multiple CRISPR-Cas nikase effectors and their ability to create a knockout in a targeted gene sequence.

Prime editing

This system involves the use of a prime editing guide RNA (pegRNA), nCas 9 (Cas9 nikase) attached to a reverse transcriptase.[33] The pegRNA will have an edited RNA segment for the knock in and a primer binding sequence.[33] The pegRNA guides the nCas 9 to the site of interest, a nick is made and the edited RNA segment to be incorporated is reverse transcribed by the reverse transcriptase and ultimately incorporated into the DNA segment.[33] Prime editing can introduce small insertions and deletions in addition to point mutations (Figure 6).[34]

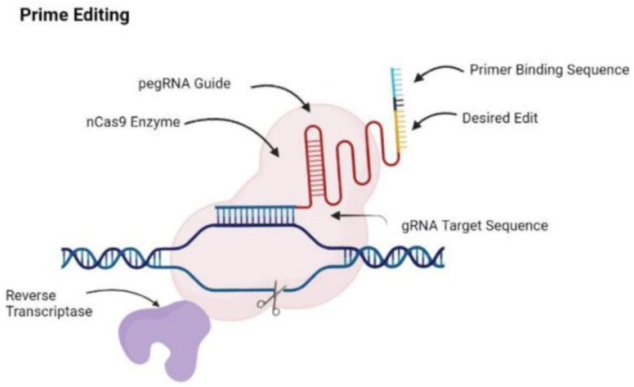

Figure 6 Illustration of prime editing.[61]

The diagram shows how prime editing involves a reverse transcriptase to knock in a gene sequence of interest.

Base editing

This strategy involves the fusion of Cas9 to nucleotide modifying enzymes for cytosine or adenine (Figure 7).[35] The main types are cytosine base editors and adenine base editors.[17] The CBE (cytosine base editor) will involve a cytosine deaminase attached an nCas9 and convert cytosine into uracil as the nCas9 nicks the double DNA strand.[36,37] Then the body will create a new DNA strand using the newly created uracil strand as the template.[2] The uracil gets converted into thymidine as the strand is repaired through natural cellular mechanisms.[2] In the case for adenine base editing, the adenine deaminase will convert adenine into inosine before the body recognizes this and changes it to cytosine [11]. An advantage of base editing is that the modification of both dividing and non-dividing cells can be done.[2]

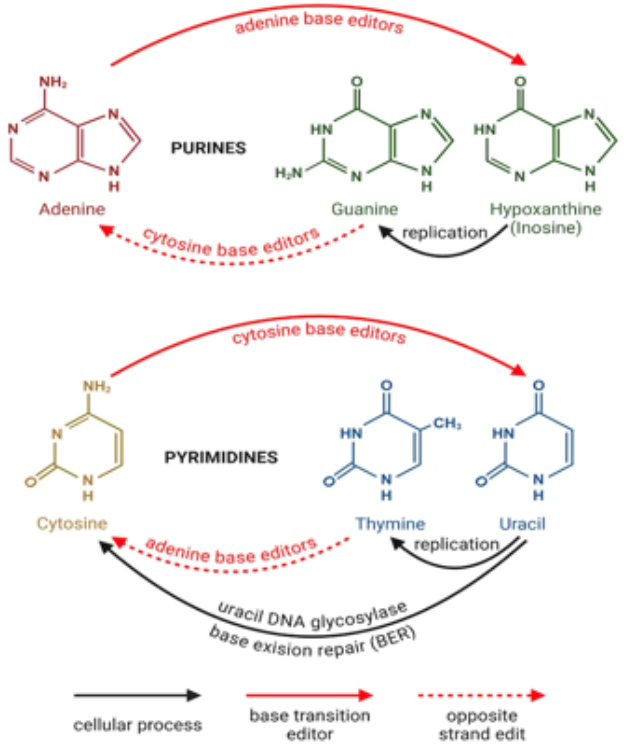

Figure 7 Illustration of base editing.[62]

The diagram shows how base editing works by using a deaminase that is attached to a Cas enzyme for base conversion.

Regulation

The gene regulation method involves either a dCas9 with a 20-nucleotide spacer combination within the sgRNA or a truncated 15 nucleotide spacer dead sgRNA.[17] These two systems may bind to a specific locus on the genome to modulate gene expression.[38] Mutations to Cas9 or Cas12a in their amino acids stops their ability to cleave but instead can be used to prevent RNA polymerases from binding their targeted open reading frame sections.[39]

Strategies for efficient gene editing

There are some methods to increase the efficiency for gene editing. This includes spacer modification, gRNA scaffold engineering, multiplexing gRNAs, chemical modification, the use of RNA aptamers,[20] and engineering circular gRNAs.[24]

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Spacer length

The first method is shortening the length of the spacer region for better specificity.[40] In the case of SpCas9, when the spacer region is shortened to 17-18 nucleotides, the CRISPR-Cas9 system becomes more specific to your target of interest.[40] It is surmised that mismatches by 2-3 nucleotides prevents off target binding.[40] If these spacer regions are reduced to 15 nucleotides, the endonuclease become dRNA (dead sgRNA), which no longer cleaves the target genes, but will still bind to those areas (Figure 8).

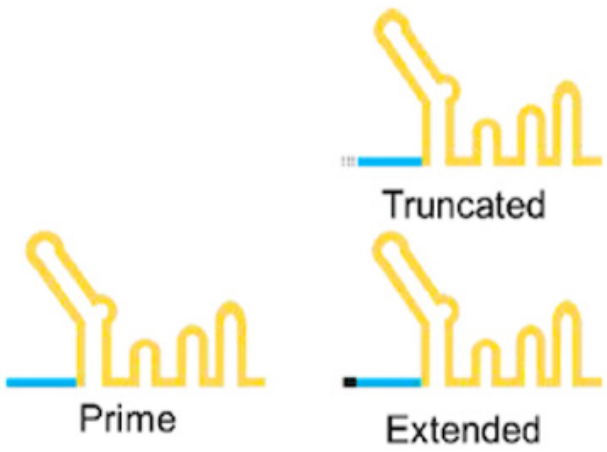

Figure 8 Figures of various spacer modifications to improve gene targeting.[8]

The diagram shows various strategies to improve gene editing through shortening or extending guide RNA spacers.

Scaffold modification

A modification of the scaffold section of guide RNAs can improve gene editing or modification.[41] Modifications in the sgRNA can stabilize and enhance dCas9 assembly through an A-U base flip in the Pol-III terminator. A Pol-III terminator is four consecutive U's and an A-U base flip in this section can avoid a premature U6 Pol-III transcription termination. Furthermore, a tetraloop extension, such as increasing the dCas9 binding hairpin structure in the sgRNA can enhance the stability of the dCas9/sgRNA complex.[41]

Multiplexing guide RNAs

Multiplexing gRNAs on a single plasmid is another way of increasing efficiency when looking to remove a section of a genome or targeting multiple sites on a genome at once.[42] Off target risks can also be reduced through the use of gRNA multiplexing.[43] Multiple gRNAs can be expressed with the use of only one promoter, either Pol II or Pol III, streamlining the gene editing process.[44]

Chemical modification

Chemical modification at the 2'OH site by 2'-O-methyl can improve site binding by increasing the T_M (melting temperature), hybridization, and sgRNA stability.[45] If a 2',4'-bridged nucleic acid N-methylated is applied to the 2'-OH sites on the ribose, then off target DNA cleavage is decreased.[46]

RNA aptamers

RNA aptamers can also be used to attract the Cas9 protein when they are added to the tetraloop portion of the gRNA.[20] The aptamers can help in gene regulation expression, precise gene editing, or live imaging.[20]

Circular guide RNA

Guide RNAs are susceptible to exosomes in their current forms and can lose their activity within 8 hours.[47] However, if guide RNAs were synthesized in a circular form with a 251 base pair insert, they would become more resistant to exosomes and maintain their function for 24 hours at 37 ^{0}C. Because circular guide RNAs last longer, they can increase the gene editing efficiency for Cas12a and Cas13d systems by being more available during the editing process.[24] Subsequently, it has been demonstrated that cgRNA can also help Cas12f to increase adenine base editing efficiency by 1.2 to 2.5 fold for similar reasons.[48]

CRISPR-Cas products in clinical trials

From December 2022, there have been 45 ongoing or completed trials involving CRISPR-Cas systems for human therapeutic product development for diseases such as HPV, hereditary angioedema, Duchenne muscular dystrophy, and many others.[35] A few of the clinical trials are described below (Figure 9).

Conditions Targeted	Gene Target	Edit Type	Therapeutic	Sponsor	Clinical Trial ID
Sickle cell disease or β-thalassemia	BCL11A	KO (NHEJ)	exa-cel	CRISPR Therapeutics and Vertex Pharmaceuticals	NCT03655678 | NCT05477563 | NCT03745287 | NCT05356195 | NCT05329649 | NCT04208529
			ET-01	EdiGene (GuangZhou) Inc.	NCT04925206 | NCT04390971 |
			BRL-101	Bioray Laboratories	NCT05577312
	β-globin	HDR	nula-cel	Graphite Bio, Inc	NCT04819841 |
			CRISPR_SCD001	UCLA, UC Berkeley	NCT04774536 |
			iHSCs with corrected β-globin	ALLIFE Medical Science and Technology	NCT03728322 |
β-thalassemia	γ-globin promoter	KO (NHEJ)	BRL-101	Bioray Laboratories	NCT04211480
Sickle cell disease			EDIT-301	Editas Medicine, Inc.	NCT05444894 | NCT04853576 |
Type 1 diabetes	proprietary		VCTX210A	CRISPR Therapeutics and ViaCyte	NCT05210530 |
Leber congenital amaurosis 10	CEP290		EDIT-101	Editas Medicine, Inc.	NCT03872479 |
Hereditary angioedema	KLKB1 (liver)		NTLA-2002	Intellia Therapeutics	NCT05120830 |
Duchenne muscular dystrophy	Dp427c	Exon skipping	CRD-TMH-001	Cure Rare Diseases, Inc	NCT05514249

Figure 9 List of current CRISPR-Cas related clinical trials.[16]

This table displays the current clinical trials involving CRISPR-Cas systems.

Type 1 Diabetes

In this 2022 trial, two companies, ViaCyte and CRISPR Therapeutics, made a combination therapy called $VCTX_{210}$ which contained a transplantable device with pancreatic beta-cell precursors called CyT49 cells.[49] These cells were genetically modified to prevent triggering an autoimmune response. These modified CyT49 cells were modified using a CRISPR-Cas9 scheme.[49]

Leber congenital amaurosis 10

Leber congenital amaurosis is a disease caused by mutations in the CEP290 gene and leads to a A-G mutation in an intron site. This mutation affects the retinal and lead to blindness.[50] This trial involves a therapeutic called EDIT-101 by Editas Medicine. The strategy involves a subretinal injection containing DNA for Cas9 and two guide RNAs using the AA5 virus. The gRNAs will target regions flanking the intronic mutation and excise it.[51]

Hereditary angioedema

This trial uses a therapeutic to attempt to resolve a life-threatening genetic disease involving a mutated gene called KLB1, which codes for prekallikrein.[52] This in vivo trial tests the efficacy of a therapeutic

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called NTLA-2002. The therapeutic is developed by Intellia Therapeutics and is intravenously delivered by a lipid nanoparticle containing Cas9-mRNA and the sgRNA for the gene KLB1.[52] NTLA-2002 will cause indels for KLB1, rendering it ineffective.[53] Hereditary angioedema can cause swelling and pain around the body.[16]

Duchenne muscular dystrophy

Duchenne Muscular Dystrophy (DMD) is a rare disease that only affects 1 in 3500 males in the world. This disease is due to a lack of dystrophin protein from frameshift mutations.[54] This stage one study involves a single intravenous injection of CRD-TMH-001 where the CRISPR mediated drug would target one or more exons that were causing frame shift mutations through exon skipping to stop the dystrophin isoforms from being produced.[16]

HIV

This in vivo trial involves a therapeutic called EBT-101, created by Excision Biotherapeutics, that is delivered using a viral vector. This therapeutic will target the HIV1 proviral DNA that has integrated into the host cell and cut out sections of the HIV1-proviral genome to stop its replication.[55]

Human papilloma virus

This is an in vivo trial associated with First Affiliated Hospital in Guangzhou, China where the CRISPR-Cas9 based therapy is encoded into a plasmid that would be introduced using a gel.[56] This therapy targets two HPV genes to knock out E6 and E7 HPV oncogenes in hopes that it will stop the growth of HPV and related cervical neoplasia.[16]

Viral keratitis, herpes simplex virus 1

This trial involves BD111 which is an in vivo CRISPR-Cas9 mRNA therapy for corneal inflammation caused by HSV-1 infection.[16] BD111 is delivered into the cornea through lentivirus as a delivery method.[16] BD111 is made to inhibit viral replication through an INDEL in the virus's genome (Figure 10).[57]

Condition Targeted	Gene Target	Edit Type	Therapeutic	Sponsor	Clinical Trial ID
HIV-1	HIV proviral DNA	Viral genome split (NHEJ)	EBT-101	Excision Biotherapeutics	NCT05144386
					NCT05143307
HPV	E6/E7 genes of HPV16/18	Viral genome split (NHEJ)	Talen: TALEN-HPV16 E6/E7 or TALEN-HPV18 E6/E7; CRISPR-Cas9: CRISPR/Cas9-HPV16 E6/E7T1 or CRISPR/Cas9-HPV18 E6/E7T2	First Affiliated Hospital, Sun Yat-sen University	NCT03057912
Viral keratitis	HSV-1 genome	Viral genome split (NHEJ)	CRISPR/Cas9 mRNA	Shanghai BDgene Co., Ltd.	NCT04560790

Figure 10 List of CRISPR-Cas associated viral clinical trials.[16]

This table shows CRISPR-Cas therapeutics involved with virus associated diseases.

Discussion

Since the discovery of CRISPR-Cas systems, researchers have been able to create a plethora of applications which include multiplex editing, gene regulation, knock out mice, and discovering genetic interactions.[2] This field does have challenges that include development and manufacturing costs which may prevent products from being scaled.[2] One such product is Casgevy, which treats sickle anemia , but costs 2.2 million USD per patient.[58] This expensive price tag may limit the number of patients the accessibility to this therapeutic.[58] Another challenge is the cost of regulations and manufacturing.[2] CRISPR-Cas therapeutics often use viral vectors and the culture systems to produce these vectors at scale can be very expensive.[59] Technical efficiency is another area within CRISPR-Cas that needs improvement. Base editing has a success rate of between 35% to 50%[11] and prime editing has a very low success rate between 0.7% to 5.5% editing rate for point mutations or transversions.[10] These inefficiencies at the technical level may contribute to the high costs for therapeutic manufacturing of CRISPR-Cas products.[2] Researchers are constantly investigating methods to ameliorate the issues with editing efficiency that includes the use of RNA aptamers on sgRNA to improve recruitment of effectors, or the modification of the 5' and 3' ends of the sgRNA which can improve specificity and efficiency.[20] Improvements in prime editing have been demonstrated through the removal of reverse transcriptase (RT) RNase H domain and adding a viral nucleocapsid (NC) protein to nCas9, which increased editing efficiency in rice and wheat.[60,61] Furthermore, new CRISPR variants that allow PAM flexibility such as xCas9 3.7 have a broad range of PAM sites to be edited, this may help improve editing efficiency for future therapeutics.[17]

Conclusion

CRISPR-Cas system therapeutics appear to have an upward trajectory.[16] As of 2022, there were around 71 clinical trials that involved CRISPR-Cas systems with 45 that were completed or still active.[16] The market share for CRISPR-Cas products can potentially reach 16.55 billion USD by 2034.[12] Advancements in gene editing, live cell imaging, and the rise of machine learning, it is possible that affordable gene therapeutics will become more common through the intersection and integration of these new technologies.[2] Researchers also have access to computer design tools such as CHOPCHOP, E-CRISP, and Synthego to facilitate improved guide RNA designs that may increase gene editing efficiency (Figure 11).[10] CRISPR-Cas systems are an important method to combat human diseases and can be used in multiple ways such as gene editing, gene deletions, and gene regulation. It is likely Casgevy will be the first of many CRISPR-Cas therapeutics to come onto the healthcare marketplace.

Toolar	Input	Web site
CHOPCHOP	DNA sequence, gene name, genomic location	https://chopchop.cbu.uib.no
CasOFFinder	DNA sequence	http://www.rgenome.net/cas-offinder
E-CRISP	DNA sequence, gene name	http://www.e-crisp.org/E-CRISP/designcrispr.html
CRISPRscan	DNA sequence	https://www.crisprscan.org
Benchling	DNA sequence, gene name	https://www.benchling.com/crispr
CRISPROR	DNA sequence	http://crispor.tefor.net
CRISPR-ERA	DNA sequence, gene name, or TSS location	http://crispr-era.stanford.edu
Synthego	Gene name	https://design.synthego.com/#
BE-Designer	DNA sequence	http://www.rgenome.net/be-designer

Figure 11 List of software's to aid in guide RNA designs.[20]

This table is a display of available web-based tools for guide RNA design.

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Conflict of interest

Authors declare that there are no conflicts of interest.

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