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Review Article

CRISPR-mediated gene editing for the surgeon scientist

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ABSTRACT

Tremendous advances have occurred in gene editing during the past 20 years with the development of a number of systems. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)–associated protein 9 (Cas9) system represents an exciting area of research. This review examines both the relevant studies pertaining to the history, current status, and modifications of this system, in comparison with other gene-editing systems and future applications, and limitations of the CRISPR-Cas9 gene-editing system, with a focus on applications of relevance to the surgeon scientist. The CRISPR-Cas9 system was described initially in 2012 for gene editing in bacteria and then in human cells, and since then, a number of modifications have improved the efficiency and specificity of gene editing. Clinical studies have been limited because further research is required to verify its safety in patients. Some clinical trials in oncology have opened, and early studies have shown that gene editing may have a particular role in the field of organ transplantation and in the care of trauma patients. Gene editing is likely to play an important role in future research in many aspects of the surgery arena.

Introduction

Substantial advances have occurred in gene editing (or the process of gene insertion, functional deletion, replacement, or modification within a given segment of deoxyribonucleic acid [DNA]) during the past 20 years, and a number of techniques have been developed. These techniques include zinc-finger nucleases (ZFN),[1] transcription activator–like effector nucleases (TALEN),[2] and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)–associated protein 9 (Cas9).[3] Each of these types of systems can create breaks in double-stranded DNA.[1–3] The repair of breaks in double-stranded DNA is accomplished principally by 2 mechanisms: nonhomologous end-joining and homology-directed repair. In the absence of a specific inserted DNA template, the break in a chromosome can be repaired by nonhomologous end-joining, which joins the broken ends of DNA together.[4] Typically, this introduces random, small insertions or deletions, also known as "indels." This process can lead to a frameshift mutation and can subsequently knockout gene function.[4] In contrast to nonhomologous end-joining, homology-directed repair is a DNA template-dependent repair mechanism of breaks in double-stranded DNA, which can create specific deletions, insertions, or substitutions.[5,6] The CRISPR-Cas9 system was proposed by Jinek et al[3] in 2012 after they observed that certain genes, which are consistently expressed in bacteria and archaea, could be engineered to create selectively a break in double-stranded DNA at a specific DNA locus targeted by a specific RNA sequence. Since then, numerous modifications of this system have been developed to improve the specificity of the CRISPR-Cas9 system of gene editing.

A number of reviews on gene editing have been published with varying levels of complexity[7,8] and targeted toward medical specialties, such as dermatology,[9] ophthalmology,[10] and cardiology,[11] but few reviews focus on general surgery or its subspecialties.[12,13] We review the history, mechanism of action, modifications, current and future applications, and limitations of the CRISPR-Cas9 system and compare this system with other systems used to study gene function. The references highlighted in this review describe some of the important breakthroughs in CRISPR-Cas9 gene editing. We believe that the CRISPR-Cas9 system will become an integral part of surgical research and will be an important addition to the tools of surgeon scientists when studying and treating diseases.

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History of CRISPR: From bacterial immunity to a novel gene-editing technology

In the final paragraph of their 1987 report, Ishino et al[14] observed an unusual repeated sequence of 29 nucleotides in the 3' end of a gene they were investigating. This repeated sequence was separated by a fixed length of different nucleotides. It was not until 2002, when the term "Clustered Regularly Interspaced Short Palindromic Repeats" (CRISPR) was coined that it was determined that these intervening segments of DNA, named "spacers," were derived from the DNA of different bacteriophages.[15–17] Koonin et al[18] first proposed that CRISPR played a central role in bacterial immunity against viruses. DNA from invading viruses is incorporated into the bacterial genome at a CRISPR locus. Three types of CRISPR-Cas systems, each with different biogenesis pathways, have been described in depth elsewhere.[19–21] The type I and III CRISPR-Cas systems are dependent on a CRISPR-specific endoribonuclease to process the CRISPR RNA (crRNA) transcripts. The type II CRISPR-Cas system uses cellular RNase III to process crRNA.[19,22] The type II CRISPR-Cas system was employed by Jinek et al[3] for RNA-programmable gene editing, which is dependent on trans-activating crRNA (tracrRNA), crRNA, and Cas9 components for gene editing. An illustration of the type II CRISPR-Cas system is presented in Fig 1. Incorporated viral DNA is transcribed into precursor crRNA (pre-crRNA), where each crRNA sequence is specific to a given viral DNA. The pre-crRNA forms a duplex with a tracrRNA.[3,22] This complex is processed by cellular RNAase III in the presence of Cas9 and by other ribonucleases to produce a mature tracrRNA-to-crRNA duplex that is bound to Cas9.[22] The tracrRNA-to-crRNA duplex acts to guide the entire tracrRNA-to-crRNA and Cas9 complex to the invading complementary viral DNA. In addition, there is a short nucleotide sequence (NGG, in the case of *Streptococcus Pyogenes*, where N can be any nucleotide and G is guanine), which is necessary for DNA cleavage.[23] This trinucleotide is known as the protospacer adjacent motif (PAM). The Cas9 complex functions to cleave the invading viral DNA at this target.

Mechanism of action of the CRISPR-Cas9 gene-editing system and modifications

In 2012, Jinek et al[3] clarified the biochemistry of this system and demonstrated programmable, targeted DNA cleavage in bacterial cells. They demonstrated that Cas9 can cleave DNA and is guided to the target DNA sequence by the 2 RNA molecules tracrRNA and the crRNA. The crRNA is a 17-20 base sequence that confers specificity in targeting the Cas9 nuclease to the target DNA sequence, but the crRNA alone is unable to cause Cas9-mediated DNA cleavage.[3,24,25] Both the tracrRNA and crRNA together are necessary for Cas9-mediated DNA cleavage. As already discussed, an additional short nucleotide sequence, the PAM, is necessary for the CRISPR-Cas system to function.[23,25] This sequence is specific to the bacterial species from which the Cas9 is derived.[26] Truncating the native tracrRNA and crRNA facilitates Cas9-mediated DNA cleavage. In a further step, a chimeric tracrRNA-to-crRNA duplex was synthesized that facilitated Cas9-mediated DNA cleavage. Cong et al[27] commercialized and optimized the CRISPR-Cas system for use in human cells. Synthesis of the chimeric tracrRNA-to-crRNA complex as a single guide RNA (gRNA) yielded the same effect on DNA cleavage in human cells. Multiple guide sequences could also be used to facilitate simultaneous editing of multiple genomic sites.[25,27] The efficiency of indel formation by a genomic cleavage assay demonstrated efficiencies of approximately 30%.[27] Further modification of the length of the tracrRNA sequence led to an increased rate of indel formation of approximately 52%.[28] A schematic of the basic mechanisms of nonhomologous end-joining and homology-directed repair are presented in Fig 2.

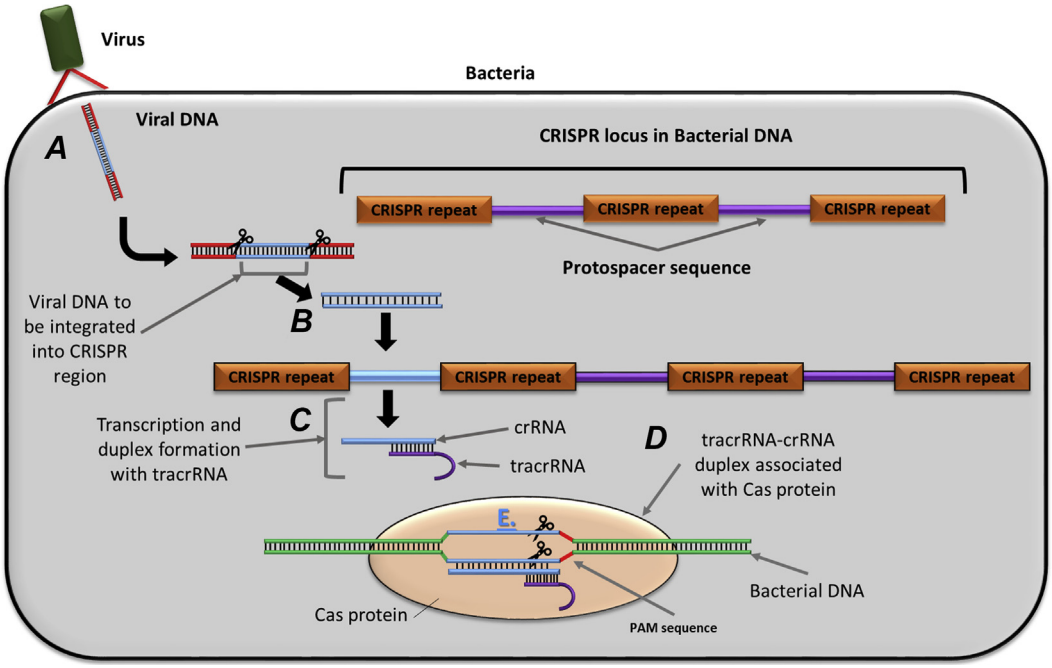

Fig 1. The mechanism of action of the CRISPR-Cas system in bacteria as an immune mechanism against viruses. (*A*) Viral DNA enters the bacterial cell when infected with a virus. (*B*) The viral DNA is integrated into a CRISPR locus of the bacterial DNA and is surrounded by CRISPR repeats. (*C*) The premature crRNA (pre-crRNA) is transcribed, associates with a tracrRNA, is processed into a mature crRNA by cellular RNAase III, and forms the tracrRNA-crRNA duplex. (*D*) The tracrRNA-crRNA duplex associates with a Cas protein. (*E*) The tracrRNA-crRNA duplex or Cas protein complex identifies regions of viral DNA within the bacterial DNA and, through the endonuclease activity of the Cas protein, removes these regions of viral DNA. The tracrRNA-crRNA duplex/Cas protein complex also requires the PAM sequence to be present for the nuclease function to occur.

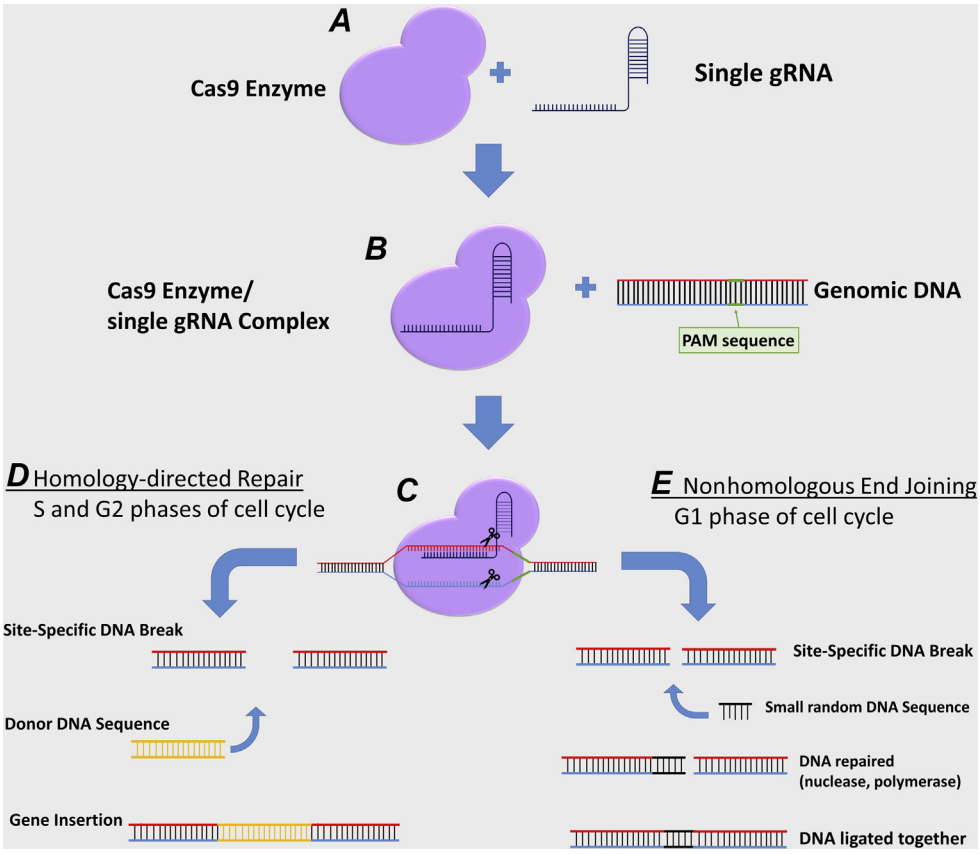

Fig 2. The mechanism of action of the CRISPR-Cas9 system. (*A*) The Cas9 enzyme combines with a single gRNA. (*B*) They combine to form a single gRNA-Cas9 complex. The PAM sequence is necessary for the single gRNA-Cas9 complex to recognize the target DNA sequence and for the nuclease activity to occur. (*C*) This gRNA-Cas9 complex targets the DNA sequence complementary to the gRNA sequence. (*D*) The gRNA-Cas9 complex binds to the DNA at this sequence and makes a DNA double-stranded break. The DNA double-stranded break can be precisely repaired with homology-directed repair (during the S and G2 phases of the cell cycle), in that a donor DNA sequence is inserted into the host DNA at the site of the double-stranded DNA break. (*E*) The DNA double-stranded break can be repaired imprecisely with the nonhomologous end-joining (during the G1 phase of the cell cycle). This creates an indel, (a small random DNA sequence can be inserted or deleted), which can cause a "functional gene deletion."

Since the initial description in 2012, the CRISPR-Cas9 system has undergone many subsequent modifications in an attempt to improve both the specificity and the efficiency.

Off-target effect

A well-recognized concern with CRISPR-Cas technology is the "off-target effect." As the DNA recognition sequence of a gRNA is relatively short (up to 25 base pairs in length) and mismatches of up to 3 bases have been tolerated,[29] there is a recognized risk for an off-target effect.[30,31] This occurs when the nucleotide sequence of the gRNA, which guides the gRNA-Cas9 complex, targets the complex to a sequence with which it does not fully complement. This off-target effect leads to unintended indel formation in the case of nonhomologous end-joining and, of special relevance and importance, the insertion of a gene in homology-directed repair to an unintended place in the genome. It is therefore critical to identify other sites in the genome that potentially could be targeted in an unintended way by a particular gRNA.[32] There is, therefore, considerable interest in the need to assess for off-target CRISPR-Cas activity and to minimize this potential off-targeting by using high-throughput sequencing. One such method is the detection and mapping of breaks in the double-stranded DNA at a nucleotide-level resolution in a procedure referred to as BLESS (direct in situ ***B***reaks ***L***abeling, ***E***nrichment on ***S***treptavidin, and next-generation ***S***equencing).[33] Although tools such as the Basic Local Alignment Search Tool can be used to check gRNA sequences for other possible complementary binding sites, more sophisticated systems have been developed.[34–36] Approaches using techniques in bioinformatics have been developed to identify sites of breaks in the double-stranded DNA in an unbiased manner for a gRNA.[37] There are also scoring systems that can predict the off-target effects for a given gRNA sequence.[28,38] Published data and commercial sources regarding the possible off-target effects of gRNA sequences both should be consulted when conducting research using CRISPR-Cas9 techniques to minimize the editing of unintended sites.

Modifications of the CRISPR-Cas9 system

The CRISPR-Cas9 system continues to be modified to improve specificity and efficiency. One such change is the use of a modified Cas9 enzyme that produces a single cut in the DNA.[27] It has been shown that each of the two catalytic protein domains in Cas9 were responsible for cleavage of one of the DNA strands.[3,27,39] Through inactivation of one of these Cas9 endonuclease domains through an amino acid substitution, Cas9 could have a "nickase" function(Cas9n) that creates single-strand breaks and facilitates homology-directed repair.[3,24,27] This process has been modified to improve the specificity of the CRISPR-Cas9 system by using different single gRNAs with a Cas nickase in a process called "double nicking," which markedly reduces off-target activity without reducing the desired on-target activity.[39] In this case, the use of paired guide RNAs and the modified Cas9 enzyme led to a

decrease in off-target activity by 50-fold to 1,500-fold in both cell lines and mouse embryonic cells.[39] Another modified application uses a catalytically dead Cas9 (inactivation of both endonuclease domains) to target a transcriptional activator or repressor domain, thus facilitating the modulation of gene expression.[40,41]

The rates of homology-directed repair are quite variable between different cell types.[25] Targeting rates between 10% to 25% were observed in human embryonic kidney HEK293T cells, from 13% to 38% in human chronic myelogenous leukemia K562 cells, from 2% to 4% in human-induced, pluripotent stem cells, and in up to 80% in mouse embryonic cells.[25,42] Because nonhomologous end-joining is the main DNA repair pathway in mammalian cells,[43] various molecules have been investigated to increase the efficiency of homology-directed repair. Small molecules have been shown to increase gene editing efficiency by a magnitude of 2- to 3-fold in porcine fibroblasts.[44] The selection of certain genomic locations have been demonstrated to produce consistent transgene expression, such as adeno-associated integration virus 1(AAVS1).[45] A limitation of current homology-directed repair technology is the size of the template DNA insert. In addition, concerns about abnormal chromosomal recombination in homology-directed repair also need to be addressed with CRISPR-Cas9 gene editing.[46]

The development of a single, base-editing system has also been developed, whereby an adenosine or cytidine deaminase is fused to a catalytically inactive Cas9 enzyme to mutate adenosine or cytidine bases to functional guanosine or thymidine bases.[47–49] This technology has the potential to mutate single base pairs involved in diseases, without creating breaks in double-stranded DNA in the genome. This may have substantial potential clinical benefit in the future.[48] Other such innovations with the CRISPR-Cas9 systems are the engineering of chromosomal translocations[50] and entire chromosomal deletions in vivo.[51]

System delivery to cells

One of the major issues with current CRISPR-Cas9 technology is delivery of this system to target cells.[52,53] Both viral and nonviral delivery vectors and physical methods of delivering CRISPR-Cas9 systems into cells allow for the delivery of mRNA products or plasmids containing the CRISPR-Cas9 system.[52] Adeno-associated viruses (AAV) have been described as attractive vehicles for system delivery,[54] but viral vectors are restricted by the cargo size ($\approx$ 4.5 kb) they can accommodate. The use of *Streptococcus Pyogenes* Cas9 (SpCas9) ($\approx$ 4.2-kb cargo size) is challenging and limits further modification of the CRISPR-Cas9 system.[55,56] The use of *Staphylococcus aureus* Cas9 (SaCas9), however, is a potential solution because of its smaller size ($\approx$ 3.1 kb), which facilitates the delivery of the CRISPR-Cas9 system into cells.[57] Ran et al[57] demonstrated that SaCas9 and a single gRNA could be packaged into a single viral vector to regulate gene expression. The use of SaCas9 may allow for the CRISPR-Cas9 system to be packaged more easily into a viral vehicle for transfection. It is important to note, however, that the PAM sequence differs between SpCas9 and SaCas9. This is an important determining factor in which genomic locations the CRISPR-Cas9 system can function.[3,25,27] Physical delivery systems, such as electroporation or microinjection, have been described for the CRISPR-Cas9 system[58] and may have an advantage over viral-mediated transfection because of the described risk of carcinogenesis and immunogenicity with viral-mediated transfection.[59–61]

Cell cycle effect

Another major limitation with the current CRISPR-Cas9 system is the influence of the cell cycle on the gene-editing process. The process of homology-directed repair with the CRISPR-Cas9 system is dependent on proteins that are expressed preferentially in the S and G2 phases of the cell cycle, whereas nonhomologous end-joining occurs during the G1 phase of the cell cycle.[4,62,63] Recent reports suggest that CRISPR-mediated genome editing can lead to p53-mediated DNA damage and induce cell cycle arrest.[64] In addition, homology-directed repair selects for cells without a functional p53 pathway, thereby promoting the survival of a population of cells without p53 signaling activity.[64] This process has the potential for leaving cells vulnerable to chromosomal rearrangement and other tumorigenic changes.[64] Further work is essential to delineate this relationship to ensure that gene editing occurs with a minimal production of potentially tumorigenic cells. This possibility represents a major barrier for the transition of CRISPR into clinical studies.

Comparison with Other Systems to Study Gene Function

An in-depth description and comparison of the various systems to study gene function have been described by Boettcher et al.[65] Some of the differences between the gene-editing systems are described in Table I. Numerous commercial biotechnology companies now offer consultation services to implement various systems in investigators' laboratories (eg, ThermoFisher Scientific, Sigma Aldrich, Origene, GeneCopoeia, etc; Table II).

RNA interference

RNA interference (RNAi) is a widely used approach for studying gene function in mammalian cells and is accomplished most commonly using short interfering RNAs or with short hairpin RNAs.[66,67] This technique facilitates knock-down of cellular RNA expression through a posttranscriptional mechanism that can lead to the subsequent regulation of other molecules.[68] In clinical practice, short interfering RNAs have been used in trials as a treatment for selected hepatic diseases and hepatic fibrosis.[69] There are some important issues with using RNAi to study gene function. Depending on the siRNA sequence, specificity can vary substantially because numerous transcripts can be repressed.[70] Viral transductions (eg, lentiviral-mediated short hairpin RNAs) increase the chance of activation of oncogenes.[71] Regulation of some cellular molecules can be challenging with RNAi. RNAi principally occurs in the cytoplasm, and for molecules that are located in the nucleus, regulation of gene function may be challenging, such as in the case of studying long, noncoding RNA.[72–75] As such, the use of CRISPR-Cas9 technology may be a more effective alternative for the study of these molecules.[74]

Zinc finger nuclease gene-editing system

Zinc fingers are a class of DNA binding proteins that recognize 3 to 4 base pairs of DNA,[76] and through a combination of a number of zinc fingers, target DNA sequences can be recognized with high specificity.[1] The fusion of the nonspecific endonuclease FOKI to a zinc finger produces a DNA cleavage system.[77] A pair of ZFNs are required for FOKI to mediate DNA cleavage through the dimerization of two FOKI monomers. The wild-type FOKI cleavage domain is nonspecific and, as such, does not preferentially select for heterodimerization (ie, a "left" and "right" ZFNs).[78] Modification of the FOKI monomer for each of the left and right ZFNs can confer increased specificity with decreased homodimerization.[78] This system is relatively efficient with reported gene editing rates of up to 20% in Il 2Rgamma, a gene involved in Severe Combined Immune Deficiency.[1] In another example, targeted deletion of a 32-base pair segment of DNA from the gene CCR5,

Table I
Details of gene-editing systems

Gene-editing system	Acronym	Method of DNA location recognition	Type of DNA binding	Type of nuclease	Mechanism of DNA cleavage	Efficiency	Specificity	Time to manufacture	Cost
Zinc-finger nucleases	ZFN	- Each zinc finger recognizes a DNA triplet - Each zinc-finger nuclease consists of 3-6 zinc fingers - "Left" and "right" ZFN confer specificity	Protein-DNA	FOKI	A pair of ZFNs required to target FOKI nuclease monomers to dimerize to facilitate DNA cleavage	Moderate	High	Slow	Expensive
Transcription activator-like effector nucleases	TALEN	- Each TALE consists of ≈30 tandem repeats of ≈30 amino acids - Each tandem repeat contains 2 central amino acids that have specificity for a base pair - Combining repeats confers specificity - "Left" and "right" TALEN confers specificity	Protein-DNA	FOKI	A pair of TALENS required to target FOKI nuclease monomers to dimerize to facilitate DNA cleavage	Moderate	High	Slow	Affordable
Clustered regularly interspaced short Palindromic repeats-CRISPR-associated protein 9)	CRISPR-Cas9	- gRNA recognizes DNA sequence* - A PAM sequence must be present in the bases upstream of the target DNA sequence	RNA-DNA	Cas9	gRNA targets Cas9 nuclease to cleave DNA at the target DNA site	High	Moderate	Fast	Affordable

* General form of target DNA sequence that is necessary for gRNA to bind—for example, for Staphylococcus Pyogenes Cas9, spCas9- $G(N)_{20}GG$, where the protospacer adjacent motif (PAM) sequence consists of the final 3 nucleotides (N)GG. N = any nucleotide; $(N)_{20}$ = designed gRNA sequence. The PAM sequence is a specific nucleotide sequence upstream to the target DNA sequence, which is essential for guide RNA binding.

Table II
Description of services offered by commercial vendors

CRISPR-Cas9	
ThermofisherScientific.com	Custom gRNA and library of gRNAs DNA, mRNA, and protein delivery products
Sigmaaldrich.com	Custom gRNA and library of gRNAs DNA, mRNA, and protein delivery products
Dharmacon.horizondiscovery.com	Custom gRNA and library of gRNAs DNA, mRNA, and protein delivery products
Origene.com	Custom gRNA DNA, mRNA, and protein delivery products
GeneCopoeia.com	Custom gRNA and library of gRNAs Cas9 stable cell lines DNA, mRNA, and protein delivery products
Addgene.org	CRISPR kits and plasmids
TALEN	
ThermofisherScientific.com	Custom TALEN
GeneCopoeia.com	Custom TALEN
Addgene.org	TALEN constructs and module plasmids
ZFN	
Dharmacon.horizondisocvery.com	Custom ZFN
Sigmaaldrich.com	Custom ZFN

using ZFNs, has been shown to confer resistance to human immunodeficiency virus (HIV) infection.[79] The CCR5 gene is essential for the integration of the HIV virus into human cells, and the deletion of this segment by ZFNs has been examined in patients with HIV.[80]

Transcription activator-like effector nuclease gene-editing system

Transcription activator-like effectors (TALE) are also a class of DNA binding proteins that have specificity for a particular DNA location, which is dependent on the composition of the individual protein domains of the TALE.[81,82] Each of the DNA-binding domains of a TALE contain upward of approximately 30 repeats, each of which is approximately 25–30 amino acids in length.[65,82] The specificity of the TALE is conferred by the amino acids at the center of each of the DNA-binding domains.[82] These amino acids are known as the "repeat variable di-residue (RVD)."[83] The transcription activator-like effectors are conjugated to the FOKI nuclease enzyme to produce a TALE with a nuclease activity (TALEN).[2] As in the case of ZFNs, FOKI requires dimerization to produce a break in double-stranded DNA, which requires the synthesis of a pair of TALENs.[6] Although there is substantial specificity conferred by the large size of a single TALEN (≈ 500–700 amino acids each), the design process can be extremely complicated.[2] As such, there is scope for homology-directed repair with the specificity conferred by paired TALENs.[84] Some biotechnology companies offer TALEN consultation services to assist in their development. TALENs are approximately 3–4 times the size of ZFNs so the method of delivery may be a particular issue if multiple TALENs are being transfected.[83] As expected, there is relatively high specificity and a decrease in the off-target effect seen with TALEN gene editing because the FOKI endonuclease is required to dimerize to induce a double-stranded break.[65,85] TALENs have been shown to have a spectrum of function depending on the configuration. In an early study by Miller et al,[83] rates of gene modification of between <1% to 27% were observed. The spectrum of efficiency is determined by a number of factors, such as different left and right TALENs and the number of bases that are between the left and right TALEN.[83]

A relative disadvantage of the ZFN or TALEN systems as compared with the CRISPR-Cas9 system is the complexity in the design and building of a pair of ZFN or TALEN molecules compared with the creation of a single gRNA for the CRISPR-Cas9 system to target a DNA sequence.[52] In the case of laboratories with limited resources, the CRISPR-Cas9 system offers the ability to use a gene-editing technique that may not have been possible previously. The relatively greater molecular weight of the ZFN or TALEN components compared with a single gRNA and Cas9 also poses difficulties with respect to delivery of the system[52,65]; however, the specificity conferred by the large sequences of the ZFN or TALEN system have clear advantages over the shorter sequence of the gRNA of the CRISPR-Cas9 system. By inactivating the nuclease domains of the Cas9 enzyme, "dead Cas9" (dCas9) is created and can then be fused to different molecules.[86] When FOKI, the same enzyme used in ZFN and TALEN technology, is fused to dCas9, a pair of gRNAs can be used to increase the specificity of DNA cleavage to >140-fold compared with wild-type Cas9.[86] The CRISPR-Cas9 system can facilitate the editing of multiple genetic locations simultaneously, which is another advantage compared with the other gene-editing systems.[25,27] Early CRISPR-Cas9 systems were able to demonstrate an effect as early as 20 hours after transfection in contrast to 40 hours after transfection with TALENs.[29] The time for transduction was further decreased with the use of an adeno-associated virus–delivered, CRISPR-Cas9 system with DNA cleavage seen as early as 3 hours.[56] As the CRISPR-Cas9 system is further improved, the observed differences between the gene-editing systems will likely become more pronounced.

Translational Research Uses

Studying diseases

The use of gene editing in hematologic disorders caused by a single gene mutation has provided an attractive first target for clinical research. For example, the CRISPR-Cas9 editing system has been used to manipulate the genome to ameliorate certain diseases models, such as in beta-thalassemia,[87] sickle cell anemia,[88] hemophilia A,[89] and polycythemia vera.[90] Correction of non-hematologic, monogenic diseases, such as cystic fibrosis,[91] Duchenne muscular dystrophy,[92] and chronic granulomatous disease,[93] have also been demonstrated in human-induced pluripotent stem cells (hiPSC). These hiPSCs are somatic cells that have been modified to overexpress certain transcription factors, allowing them to assume a pluripotent phenotype.[94] Combining these cells with CRISPR-Cas9 technology provides a good platform to study diseases caused by a specific mutation, although there are some issues with hiPSCs because they sometimes fail to demonstrate the characteristics of the cells they are directed to become.[95] In addition to studying single genes, the induction of mutations in multiple genes in normal tissue is an interesting avenue for study in tumor biology.[96] Matano et al[96] induced mutations in genes associated with colorectal cancer in normal colon mucosa cells that were grown into organoids. Of note, these organoids formed tumors in mice, with subsequent formation of micrometastases[96]; however, it was only with the use of adenoma tissue that creation of these same mutations induced macrometastases.[96] This suggests the utility of this technology for discovering novel mutations in cancer. The CRISPR-Cas9 gene-editing system allows for the "knock in" (gene insertion) and "knock out" (gene deletion) mutations in cell lines to study gain of function and loss of function of gene expression on phenotype. A TALEN-based method of correcting a gene causing dystrophic epidermolysis bullosa has been used as a method of growing skin with an epidermal-dermal junction, implying that it might have a therapeutic role in the future.[97] This observation also suggests that the CRISPR-Cas9 system could play a similar role in the future in tissue engineering.

Drug discovery

The CRISPR-Cas9 system has been used to induce mutations in a number of genes simultaneously to identify and validate genes involved in carcinogenesis.[98] Using a CRISPR-Cas9 activation screen, with the CRISPR transcriptional activator modification, a number of long, noncoding RNA loci were shown to mediate resistance to vemurafenib in a melanoma cell line.[99] Although there are limitations to using cell lines in drug discovery, the target of specific genes with CRISPR-Cas9 also allows for the validation of drug-target validation studies in vitro.[100] Selective mutation of the exportin-1 gene with CRISPR-Cas9 conferred resistance to selinexor, an anticancer drug currently being investigated in clinical trials.[100] Human iPSCs, which can be edited to express a diseased phenotype, have also been employed for drug screening, allowing for in vitro pharmaceutical testing on a model very similar to native diseased cells.[101] But, as discussed earlier, there are issues with the use of hiPSCs because they may not always demonstrate the characteristics of the desired cell type.

Clinical Trials

The first report of the CRISPR-Cas9 technology being used in a clinical trial was in China in 2016.[102,103] In this trial, a group at Sichuan University used CRISPR-Cas9 to knock out the PD-1 immune checkpoint protein in the peripheral blood T cells of a patient. The edited cells were then reinfused as a therapy for lung cancer. The first trial using the CRISPR-Cas9 system in the United States opened in January 2018 for patients with advanced multiple myeloma, melanoma, synovial sarcoma and myxoid or round cell sarcoma.[104]

Animal models using CRISPR-Cas9-mediated genome editing as a therapy for retinitis pigmentosa have been described[105] because eye diseases are recognized as being ideal targets for genome-editing therapeutics.[106] Genome-editing technologies have also been studied for the treatment of several hematologic and immunologic disorders, such as in sickle cell disease and severe combined immunodeficiencies.[107] Several cancers have been proposed for CRISPR and Cas9-based therapies, including osteosarcoma and anaplastic thyroid carcinoma.[108–110] Although there is a great deal of further research to be done to address the existing and new issues with CRISPR-Cas9 technology in vivo, system delivery may be a specific niche for surgeons for clinical trials. Gold nanoparticle delivery has been described.[111] Surgeons may have a role in the implementation of this technology.

As discussed earlier, a ZFN editing system has been used in a small investigational series of 12 patients with HIV to delete a 32 base pair segment of DNA from the CCR5 gene.[79] This approach led to a decrease in HIV RNA in most patients, with HIV RNA becoming undetectable in one of the patients in the study.[80] Parallels could be drawn in the areas of surgical infection and immune dysfunction, where numerous genes have been identified to be dysregulated in trauma and burn patients.[112,113] Again, further study is required in vitro to identify whether gene editing could be safe in vivo or if there is a potential future clinical use.

Future Applications

As CRISPR-Cas9 gene-editing technology is constantly developing and evolving, there is an expanding role for various uses in surgical diseases.

Xenotransplantation is one of the foremost areas where CRISPR-Cas9 gene editing may be of major use in bridging the gap between organ supply and demand. With more than 100,000 patients currently awaiting an organ transplant and a lesser supply of available organs, xenotransplantation research could be a major target for the CRISPR-Cas9 gene-editing technology.[114] Porcine models to grow organs for human transplantation have been studied elsewhere.[115,116] Research into porcine xenotransplantation was hampered by technical limitations, concerns about zoonotic diseases, and transplant rejection.[12] Genetically engineering pigs for xenotransplantation had largely been abandoned except for specialized research groups because it had been an arduous and expensive process. CRISPR-Cas9 gene editing has reinvigorated this field because it offers the ability to target genes more easily than earlier techniques.[117] The hope with this technology is that it may be able to produce donor pigs with markedly decreased antigenicity on a time scale of months rather than the years required previously. CRISPR-Cas9 gene editing has also shown promise in eliminating retroviral DNA from the porcine genome, which provides an interesting mechanism to potentially target the transmission of zoonotic diseases with xeno-transplantation.[118] CRISPR-Cas9 allows for the knock-out of MHC genes,[119] genes encoding carbohydrate xenoantigens,[120] and precise transgene knock-in to the pig genome.[121] This approach could produce pigs that may be more immunologically suitable for organ growth for the purposes of xenotransplantation in humans. Hyperacute rejection and acute vascular responses have led to limited graft survival in pig-to-primate xeno-transplantation. CRISPR-Cas9 genetic modifications could be a new avenue of research to decrease rejection and prolong graft survival.[117] If grafts can survive the initial host rejection, exogenous immunosuppressive therapy would in turn limit the subsequent innate and adaptive immune responses. Pancreatic islet cell xenotransplantation could become clinically available.[122,123] Research on the optimization of heart, kidney, liver, lung, corneal, and tissue xenotransplantation is currently underway.[124–131] The combination of CRISPR-Cas9 technology and xenotransplantation, although still in its early stage, is an exciting new direction.

Although CRISPR-Cas9 technology is a rapidly advancing technology, it is important that extensive in vitro experimental work is conducted before its use in patients. Despite the current advances, there are several important challenges in terms of specificity, safe delivery, and abnormal recombination that must addressed. The recent study demonstrating a selection of cells with a nonfunctioning p53 pathway poses a potential risk for carcinogenesis.[64] With a recent presentation of the use of the CRISPR-Cas9 technology to modify the CCR5 gene in human embryos, which produced the first "CRISPR babies," even ethical questions have been raised.[132,133] Off-target mutations occurring in mammalian embryos have been reported elsewhere.[134]

In conclusion, gene editing through the CRISPR-Cas9 editing system presents many new opportunities for the surgeon scientist, and many advances in this technology have been described. There is great and encouraging potential for use in translational research and many promising clinical applications; however, there is much more work to be conducted before its use in patients. Issues with off-target effects and the effect of CRISPR-mediated editing on the mutational load of a cell are still under investigation and currently cloud the transition to broader clinical application.[135] With the extent of current research and emphasis on optimization of gene-editing technology, CRISPR-Cas9 gene editing will become an important tool for the surgeon scientist.

Conflict of interest

All authors declare no conflict of interest or relevant financial disclosure.

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