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Review

Nanosurgery Myth or Miracle A Review

Jack E Kornfeld[1*] and Joseph M Rosen[1]

[1]Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire, USA

Abstract

To perform nanosurgery, precise molecular incisions need to be made at a level that is about a billion times smaller than most surgical instruments. Not long ago nanosurgery on the human genome seemed intangible, inaccessible and a therapeutic of the distant future. With the discovery of the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system and repurposing of the CRISPR associated-(Cas)-9 protein as a nanosurgeon, nanosurgery is now possible. We review advances in nanosurgery and gene editing through the lens of the CRISPR system and Cas9. The history of the CRISPR system is explored, elucidating Cas9's bacterial origins in viral warfare. Cas9's nanosurgical technique and high degree of modularity is revealed and applied to human therapeutics. Miraculously, nanosurgery is no longer a myth and Cas9 is currently being used to fix monogenic diseases in the lab and in clinical trials.

Introduction

Until recently, surgery was classically an endeavor marked by skilled hands and scalpels. Surgeons can physically see and touch afflicted tissues. But what about pathologies on the nanoscale? What about surgery of the genome? What if we could perform surgery on the genome to prevent diseases that manifest on a macroscale from ever happening? Even the most precise microsurgery techniques cannot visualize the nanoscale. The nanoscale ranges in size from a small protein to the size of a virus, which is around a billion times smaller than a surgeon's scalpel. Fortunately, there are new emerging technologies in the field of gene editing that will allow us to perform nanosurgery on the genome. These nanosurgeons are in fact proteins, similar to the molecules that allow the cells that we are made of to function. Recently, a breakthrough technology named CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) burst onto the field of nanosurgery and is now the leading ribonucleic acid (RNA) guided nanosurgeon [1]. CRISPR technology originates from an ancient acquired immune system found in bacteria and archaea [2-5]. There are many types of CRISPR associated (Cas) proteins in the CRISPR system that can be exploited for human therapies. The most common CRISPR nanosurgeon is known as Cas9. Cas9 has a well understood nanosurgical technique. For Cas9 to perform nanosurgery, it must be delivered to the correct target -cell. There are many technologies to Cas9 to target-specific cells. However, these technologies will require further development to improve accurate and safe delivery of Cas9. Cas9 has the potential to impact human gene therapy, healthcare, biotechnology and more. The first clinical trials using Cas9 began last year. The multi factorial capabilities of Cas9 make it the crux of a several billion-dollar industry and a future backbone of personalized medicine.

The CRISPR System

Since their origin, archaea and bacteria, the pioneers of life on earth, have been in constant battle with the non-living: viruses. The clustered regularly interspaced short palindromic repeats (CRISPR) system evolved in archaea and bacteria as a mechanism to fight viral infection and retain memory of past viral infections, like an acquired immune system [4-6]. Viruses infect archaea and bacteria by injecting their deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) into the host and hijacking the host cellular machinery to self-replicate [7]. The CRISPR system intercepts and stores viral nucleic acid in a three-step adaptive immunological process [8]. The first step is known as acquisition. During the acquisition phase, the viral nucleic acid injected into the host is recognized as foreign and its DNA form is inserted into the host genome flanked by repeat sequences [8]. Repeat sequences are sections of DNA with stretches of the same or alternating nucleotides. The repeat sequences serve as a marker for where to insert foreign genetic material and are where the CRISPR name originates from [8]. The foreign genetic

***Corresponding Author:** Jack E Kornfeld, Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire, USA, E-mail: Jack.E.Kornfeld.MED@dartmouth.edu

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material inserted between the repeats is known as a spacer [8]. The bacteria are now immunized against the acquired spacer.

In-text Abbreviations	
Abbreviation	**Term**
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Cas	CRISPR associated
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
crRNA	CRISPR RNA
RNP	Ribonucleoprotein
tracrRNA	Transactivating CRISPR RNA
PAM	Protospacer adjacent motif
SpyCas9	Streptococcus *pyogenes* Cas9
G	Guanine
HNH	Homing endonuclease
sgRNA	Single guide RNA
HDR	Homology directed repair
dCas9	Deactivated Cas9
A	Adenine
NHEJ	Non-homologous end joining
LPN	Lipid nanoparticles
AAV	Adeno-associated virus
DMD	Duchenne muscular dystrophy
hiPSC	Human induced pluripotent stem cell
CAR	Chimeric antigen receptor
HLA	Human leukocyte antigen
TCR	T cell receptor
GVHD	Graft versus host disease
PD-1	Programmed death-1
HPV	Human papillomavirus
CIN	Cervical intraepithelial neoplasia

Table 1: List of terms and their corresponding abbreviations in the order they appear.

The next step is CRISPR RNA (crRNA) biogenesis. During crRNA biogenesis, the newly acquired spacer and Cas protein gene cassette are transcribed into pre-crRNA and Cas proteins (pending translation), respectively. The pre-crRNA is processed into a mature crRNA via post-transcriptional modifications, which prepare the crRNA for binding Cas proteins [8]. There are three main classes of Cas proteins, Type I-III. Each class of Cas proteins fold into a unique structure around a crRNA core forming a ribonucleoprotein (RNP) complex [8]. Type II proteins such as Cas9, require another segment of RNA known as transactivating CRISPR RNA (tracrRNA). The tracrRNA binds the crRNA to form a unique RNA moiety that recruits Cas9 and stabilizes the RNP complex [8]. Interference is the final step of CRISPR mediated adaptive immunity in bacteria and archaea. The RNP complexes, such as Cas9, target and cleave viral nucleic acid with a sequence complementary to their crRNA, thus deactivating the virus [8]. It is important to note that the RNP complexes are highly specific and will only cleave viral nucleic acid if their crRNA sequence is complementary to the target viral nucleic acid sequence. Specificity is crucial for using CRISPR in the field of gene editing and nanosurgery because it is necessary to prevent off-target cleavage and disruption of the incorrect gene.

The Surgeon: Cas9 Nanosurgical Method

Cas9 is the most commonly used nanosurgeon. Thus, its structure and technique for cutting nucleic acid has been most extensively studied. An understanding of the process of Cas9 biogenesis, target searching and target cleavage is best achieved through an exploration of Cas9 structural dynamics. Cas9 has an architecture composed of two, lima bean-shaped lobes, named the nuclease lobe and the alpha helical lobe [9]. Before binding the crRNA: tracrRNA complex, the two lobes of Cas9 arrange in a conformation similar to a book opened to its halfway point laying on a table, with each lobe representing a cover of the book [9]. Cas9 recognizes a stem loop moiety formed by the crRNA: tracrRNA complex. Upon binding the crRNA: tracrRNA complex at the center of the two lobes, which is analogous to the spine of the book, Cas9 undergoes a global conformational change [9]. The conformational change is characterized by the nuclease and alpha helical lobes swinging inwards like a clamp to form a cleft that the crRNA occupies. The tracrRNA is still partly bound to the crRNA and on its opposite end is enveloped by the alpha helical lobe. The crRNA cleft is now primed for DNA scanning, searching and interference [9]. Cas9 rapidly scans DNA for the target strand sequence or protospacer and an additional component of specificity known as the protospacer adjacent motif (PAM) [10]. The PAM is a short sequence of nucleotides on the viral DNA directly upstream of the protospacer, but located on the non-target strand of DNA [10]. The PAM is recognized by the nuclease lobe of Cas9 [10–12]. The PAM sequence varies depending on the bacterial species [13]. Streptococcus *pyogenes* Cas9 (SpyCas9) is most commonly used and has a PAM sequence of 5'-NGG-3', where N means any nucleotide (adenine, guanine, cytosine or thymine) and G indicates guanine is required [10-12]. The PAM acts as a licensing factor. crRNA:tracrRNA:Cas9 will only begin to unwind and cleave target double stranded DNA if the proper PAM sequence is recognized on the non-target strand of DNA. If the PAM is recognized, the crRNA:tracrRNA:Cas9 complex will sample the protospacer sequence for complementarily to the 20 nucleotides at the 5' end of the crRNA [10,12]. This section of the crRNA, named the spacer sequence, sits in the cleft formed by the two lobes of Cas9. Complementarity of the DNA target strand to the spacer sequence will drive spacer: protospacer base pairing, thus displacing the

non-target strand. The formation of a spacer: protospacer 20 nucleotide duplex triggers a concerted conformational change in the nuclease lobe of Cas9. The nuclease lobe contains the two active sites of Cas9; the RuvC domain and the homing endonuclease (HNH) domain. The active sites are DNA nanoscissors. Each active site cleaves one strand of the DNA. The RuvC domain cleaves the non-target strand of DNA and the HNH domain cleaves the target strand of DNA [10-12].

Protein Engineering: Expanding the Nanosurgical Toolbox and its Biomedical Applications

When Cas9 began to be repurposed for gene editing technologies researchers wanted to simplify the nanosurgical process. One way they accomplished that was by fusing the crRNA: tracrRNA guide into a single guide RNA (sgRNA), while maintaining the same capabilities as the natural RNA complex [1]. This was an important upgrade to the technology because by shrinking the system from three to two components, without affecting specificity nor activity, Cas9 became easier, cheaper and more efficient to use.

Beyond the canonical Cas9 double stranded DNA cleavage mechanism, Cas9 can be manipulated to increase its functionalities. The two active sites of Cas9 allow this nanosurgeon an element of flexibility in its surgical technique. Using protein engineering methods, the RuvC or HNH active site can be deactivated. Now, Cas9 acts as a nickase, only cutting one of two strands in double stranded DNA [14]. By using two sgRNAs, specific for adjacent sequences on opposite strands, sticky ends of DNA can be generated for homology directed repair (HDR) or insertion of a gene.

Furthermore, using similar protein engineering methods, both the RuvC and HNH active sites can be deactivated. Now, the Cas9 can no longer cleave any DNA and is known as deactivated Cas9 (dCas9). dCas9 still has specificity for the sequence of its sgRNA spacer and is useful for localizing specific targets on the genome [14]. For example, dCas9 can be used to localize to an oncogene and block its transcription, thus inhibiting tumorigenesis. Also, a fluorescent protein can be fused to dCas9 to allow for visualization or reporting of a gene target.

Overall, the utility of Cas9 for biomedical applications lies in repurposing this bacterial interference complex. By redesigning the sgRNA to target sequences of DNA underlying human disease instead of viral DNA, Cas9 can be used for therapeutic applications. For use as a therapeutic, it is crucial that Cas9 has high specificity and off-target cuts are minimized to prevent unintended harm to the genome. Recently, through protein engineering methods, a modified Cas9 has been developed that reduces the stability of the spacer: protospacer 20 nucleotide duplex, eliminating any possibility of an energetically unfavorable target mismatch [15]. Furthermore, SpyCas9 (*Streptococcus pyogenes* Cas9) can be manipulated to alter the PAM recognition capability from NGG to NAAG, allowing for increased specificity and decreased PAM induced limitations in choice of targetable sequences [13]. Once Cas9 cleaves the desired gene, the cell utilizes non-homologous end joining (NHEJ) or homology-directed repair (HDR) to repair the break in the genome. NHEJ is error prone and a good approach for inducing a gene knockout in the event you want to turn off a malfunctioning gene. HDR requires co-delivery of homologous DNA with a corrected gene and is useful for restoring function to a broken gene. While efficient and specific cleavage by Cas9 is reliable, this subsequent step required for completion of the genomic surgery, using HDR in particular, is highly variable and increases the complexity of the operation [16].

Accessing the Hospital: Cas9 Cell Delivery Technologies

Just as a surgeon must locate and gain access to the correct hospital and operating room to perform surgery, Cas9 must locate and gain entry to the correct cell type to reach the genome and perform nanosurgery. To address this issue, technologies to deliver Cas9 to cells have been developed. Currently, there are two approaches to cell delivery: *ex vivo* and *in vivo*. *In vivo* delivery is particularly complicated and is a critical area of further development of Cas9 delivery therapies for humans.

In Vivo

In vivo strategies consist of an outer biomolecular structure that protects and delivers DNA coding for Cas9 and sgRNA directly into a patient. For *in vivo* strategies, it is important to consider whether or not tissue wide distribution and editing is desired. If a specific cell type needs to be targeted, for example to avoid potentially harmful editing of another cell type, ligands specific to receptors unique to the target cell type will need to be used. This is a pitfall of all gene editing techniques because it is incredibly difficult to ensure both efficient and minimal off-target-cell delivery [17]. However, rapid progress is being made in this field [18]. Two of the main strategies for *in vivo* delivery are viral vectors and lipid nanoparticles (LNPs). Here, viruses are repurposed to house the very molecules that bacteria created to destroy them. Viral vectors usually consist of adeno-associated virus (AAV) attenuated to be non-immunogenic and packaged with DNA coding Cas9 and sgRNA matching the gene target [19,20]. Viral vectors are particularly useful because they have naturally evolved methods to infiltrate tissues and inject DNA into cells. However, it is possible that viral vectors could potentially lead to long term incorporation of DNA coding for Cas9 or sgRNA into the host cell and cause unintended genomic disruptions [21]. Fortunately, these unintended consequences have not been observed in recent experiments in mice [22]. Viral vectors also require relatively high doses, which can be financially costly. LNPs on the other hand, require more engineering to protect and

deliver DNA coding for Cas9 and sgRNA to target cells. LNPs must have engineered features to avoid immune degradation, renal clearance, non-specific interactions and have the capability to extravasate from the blood stream to reach and gain entry to the desired cell [23]. LNPs typically have a core structure similar to liposomes with specific bioactive molecules on the surface and DNA coding for Cas9 and sgRNA protected inside [23-25]. LNPs have the potential to be more accepted than viral vectors due to cultural factors and the potential to be less cytotoxic and more specific. They also can confer specificity for the liver. Recently, Cas9 was delivered using LNPs to micein vivo and shown to effectively cleave episomal and chromosomal DNA [26]. Researchers, were able to demonstrate effective *in vivo* clearance of episomal hepatitis B viral DNA in mice using LNP mediated Cas9 delivery to liver cells [26]. In the same study, Cas9 delivered using LNPs successfully knocked down proprotein convertase subtilisin/kexin type 9 gene expression, a gene closely associated with hypercholesteremia [26]. The successful editing of not only chromosomal DNA, but also episomally located DNA by Cas9, further indicates the merit of versatility conferred by the Cas9 nanosurgeon.

Ex Vivo

Ex vivo delivery consists of extracting the desired cell type from a patient, editing the cell, culturing the corrected cell and implanting the corrected cells back into the patient. The *ex vivo* approach is often used for immunotherapy and stem cells. For the *ex vivo* strategy, Cas9 and sgRNA are usually delivered as a preformed RNP through electroporation, whereby cells are shocked to stimulate uptake [22]. This approach is useful for fast and transient Cas9 activity and avoids the potentially harmful effects of Cas9 DNA integration into the host genome. To enhance cellular uptake, the electroporation method can also be combined with AAV mediated delivery.

Current Cas9 Human Applications

The World Health Organization states that over 10,000 human diseases are currently known to be monogenic [27]. Monogenic diseases are the result of mutations in a single gene in every cell of the body [27]. Genetically speaking, the simplicity of monogenic diseases makes them excellent targets for the Cas9 nanosurgeon. This means that Cas9 has lots of work to do and shows the importance of nanosurgery in the future of medicine. The current pipeline for the development of Cas9 based nanosurgical therapies typically begins with animal models and human tissue culture based *in vitro* analyses, before progressing to clinical trials.

Recently, there have been many successful applications of Cas9 to *in vitro* nanosurgical correction of genetic disease. Duchenne muscular dystrophy (DMD) is the most common fatal childhood genetic disease [28]. DMD is often caused by a frame shift mutation in a gene encoding a protein named dystrophin that is necessary for stabilizing the structure of muscles cells [28]. DMD leads to muscle degeneration and death around age twenty [28]. Using Cas9, researchers performed nanosurgery *in vitro* on the DMD gene of human induced pluripotent stem cell (hiPSC)-derived skeletal and cardiac muscle cells [28]. A relatively benign segment of the gene was removed, reframing the gene, thus restoring function to dystrophin protein and stability to hiPSC-derived skeletal and cardiac muscle cells [28]. Furthermore, the researchers engrafted reframed hiPSC-derived skeletal muscle cells, successfully restoring dystrophin *in vivo* in a mouse model of DMD [28].

Another exciting application of Cas9 to genomic nanosurgery is for chimeric antigen receptor (CAR) T cell therapy. CAR-T cell therapy is a promising approach to treating cancer [29]. CAR-T cells are generated by transfecting into T cells a gene for a chimera of a T cell activation domain, co-stimulatory domain and an antibody-derived recognition moiety specific for a tumor antigen [29]. The chimera allows T cells to target cell surface proteins specific to cancer cells without the need for binding and activation by human leukocyte antigen (HLA), which is often not present on tumor cells [29]. However, normal CAR-T cells require development from self T-cells, which is time consuming, sometimes difficult to obtain and costly [30]. Researchers recently used Cas9 to remove the HLA class I and T cell receptor (TCR) genes from CAR-T cells without an effect on their antitumor activity in both a human cancer cell line *in vitro* and a lymphoma xenograft mouse model *in vivo* [30]. This is an important advancement because HLA class I and TCR are two of the main T cell surface receptors that mediate graft versus host disease (GVHD) [30]. By eliminating HLA class I and TCR, the researchers opened the door for allogeneic CAR-T cell therapy making this cancer treatment much more accessible, affordable and quicker.

The first clinical trials using Cas9 based genome editing began last year. Now there are seven total, all of which are taking place in China [31]. Six of the seven trials are attempting to use Cas9 to knockout programmed death-1 (PD-1) signaling in the context of several different types of cancer [31]. PD-1 is a T cell surface receptor exploited by cancer cells to suppress the immune system allowing tumors to proliferate [30]. The seventh trial is attempting to use Cas9 to knockout two genes that have been associated with human papillomavirus (HPV) infection and proliferation into cervical intraepithelial neoplasia (CIN) [32]. No preliminary results for any of the seven current clinical trials have been released yet.

Conclusion

With the discovery and repurposing of the CRISPR system, Cas9 RNA guided nanosurgery is no longer a myth. Instead, nanosurgery is a miracle that is repairing genomes in labs around the world.

Scientists and surgeons have bacteria, archea and evolution to thank for the CRISPR system and Cas9. The CRISPR system evolved as an acquired immunity against viral infection. There are three stages of functionality of the CRISPR system: acquisition, crRNA biogenesis and interference. There are also three main classes of Cas interference RNPs: Type I-III. The most commonly used nanosurgeon in biotechnology applications is known as Cas9 and is a Type II interference RNP. Cas9 consists of a bi-lobed architecture that undergoes a global conformational change upon binding of sgRNA and protospacer DNA. Cas9 scans target DNA for a PAM sequence, which licenses Cas9 sgRNA to unwind protospacer DNA only if it is complementary. Complementarity leads to cleavage of both strands of DNA at a specific site. The Cas9 protein is highly modular and can be engineered to deactivate one or both of its DNA nanoscissors. Furthermore, Cas9 can be engineered to change the PAM recognition sequence, increasing specificity and targeting capabilities. For biomedical approaches Cas9 is loaded with a sgRNA specific for a gene causing a disease. Cas9 cleaves this gene at a specific site, which is then acted on by DNA repair pathways, such as NHEJ or HDR.

To perform nanosurgery, Cas9 must locate and gain entry into the proper cell. Currently, there are two main approaches: *in vivo* and *ex vivo*. *In vivo* techniques most commonly utilize viral vectors to house and deliver DNA coding for Cas9 and sgRNA into target cells. Nanoparticles similar to liposomes, are also used for *in vivo* delivery. *Ex vivo* delivery requires purification of the target cell type from human tissue. Cas9:sgRNA RNP complexes are subsequently delivered through electroporation alone or in combination with delivery of viral vectors containing DNA coding for Cas9 and sgRNA. Once the cells are edited, they are re-implanted into the host.

Currently, there are many efforts to correct genetically based diseases using Cas9. Due to the novelty of Cas9 and regulatory hurdles, for the most part, these are proof of concept experiments using human tissue *in vitro* or using mouse models for *in vivo* studies. There are currently seven clinical trials using Cas9 to perform gene therapy on human tissue. Most *in vitro* Cas9 mediated repair of genetic diseases is either through editing hiPSCs or purified immune cells. To name a few: *in vitro,* Cas9 has been shown to correct DMD, provide T cell immunity to HIV infection, generate allogeneic CAR-T cells with tumoricidal activity and much more [31]. Most clinical trials use Cas9 to knockout PD-1 in T cells for immunotherapy applications to treat various forms of cancer. One clinical trial seeks to use Cas9 to delete genes that facilitate HPV maturation into CIN. Although preliminary results of these Cas9 based clinical trials remain to be released, the future holds much promise for the use of Cas9 in human gene therapy. Soon, Cas9 based therapeutics will likely be used to treat kidney and cardiovascular monogenetic diseases as well [33,34].

The miracle that is the Cas9 nanosurgeon will likely play a major role in treating genetic diseases in humans in the near future. The Cas9 nanosurgeon elegantly fills a nanoscale space that cannot be addressed by the scalpel of the classical surgeon. With future improvements and innovations in Cas9 and the field of nanosurgery, gene therapy will become increasingly accessible and efficient. Soon, scientists will likely have the capability to manufacture organs. Cas9 and nanosurgery will help form the foundation of the organ manufacturing industry. For example, if a patient has a genetically based disease that causes kidney malfunction, Cas9 will be used to edit the patients stem cells and a healthy kidney will subsequently be grown and implanted into the patient. The possibilities for Cas9 and nanosurgery are endless. CRISPR and Cas9 are part of a rapidly growing gene therapy industry currently valued in the billions. Cas9 is and will continue to revolutionize healthcare and biotechnology. Nanosurgery is no longer a myth.

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