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CRISPR-Mediated Genome Editing in the Field of Surgery: Review

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Abstract

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) associated protein 9 (Cas9) genome editing technology represents an exciting area of surgery. CRISPR/Cas9-mediated genome editing technology has been extensively applied in various cells and organisms, both *in vitro* and *in vivo*, for efficient gene disruption and gene modification. 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.

CRISPR/Cas9 genome editing technology has shown great promise for the cancer treatment, organ transplantation/regeneration, cardiovascular surgery, and neurological surgery. In this review we describe the history, mechanism, limitations and ethical concerns of CRISPR/Cas9-mediated genome editing technology, and focus on the potential applications to surgical field such cancer treatment, organ transplantation/regeneration, cardiovascular surgery, and neurological surgery.

Clinical studies for surgical filed have been limited because further research is required to verify its safety in patients. Some clinical trials in cancer have opened, and early studies have shown that gene editing may have a particular role in the field of organ transplantation and neurogenerative diseases. Gene editing is likely to play an important role in future in the surgical field.

Keywords: CRISPR/Cas9; Genome editing; Cancer; Transplantation; Vascular surgery; Neurosurgery

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Introduction

In 1987 Ishino et al. [1] discovered an unusual structure of repetitive DNA downstream from the iap gene responsible for isozyme conversion of alkaline phosphatase in *Escherichia coli*, consisting of invariant direct repeats and variable spacing sequences, and these invariant direct repeats were interspaced by five intervening variable spacing sequences. The clustered regulatory interspaced short palindromic repeats were named CRISPR. These CRISPR cassettes are located in close proximity to the CRISPR associated genes (Cas), the protein products of which have helicase and nuclease activity. The basic function and mechanisms of CRISPR/Cas9 systems in bacteria gradually have become clear. CRISPR/Cas is an adaptive defense system that might use antisense RNAs as memory signatures of previous bacteriophage infection by exploiting base pairing between nucleic acids. During the adaptation stage, resistance is acquired by integration of a new spacer sequence in a CRISPR array, while during the expression stage CRISPR arrays are then transcribed and processed into small RNAs (crRNAs) and Cas proteins. In the late interference stage, the crRNA guide Cas9 proteins to cleave complementary nucleic acids [2]. In 2012, Jinek et al. [3] demonstrated that CRIRPDR/Cas9 system could produce a mature CRISPR RNA (crRNA) and base paired trans-activating crRNA (tracrRNA), together forming a two-RNA hybrid structure. Cas9 from *Streptococcus pyogenes*, together with chimeric single guide RNA (sgRNA), functions as a programmable endonuclease. Subsequently, CRISPR/Cas9 protein-RNA complexes localize to a target DNA sequence through base pairing with sgRNA, and create a dsDNA break (DSB) at the locus specified by sgRNA [4]. A catalytically deactivated Cas9 is a programmable DNA-binding protein that can turn targeted genes on and off [5]. In the prototypical type II system, the cells express the transacting crRNA (tracrRNA) and CRISPR RNA (crRNA) that encodes the spacer sequence. TracrRNA and crRNA associate with each other and coordinate with Cas9 to recognize a Protospacer Adjacent Motif (PAM) and nearby protospacer sequence, with the guidance of the

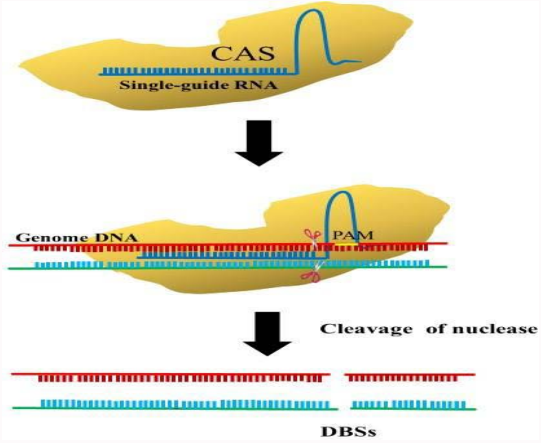

Figure 1: The mechanism of CRIPSR/Cas9-mediated genome editing. CRISPR/Cas9 is composed of Cas9 protien and a single-guide RNA (sgRNA). The (SgRNA). The sgRNA guides the Cas9 nuclease ti specific geonmic sequences *via* standard base pairing. Then, Cas9 nuclease introduces Double Stranded Breaks (DSBs) close to the Protospacer Adjacent Motif (PAM).

spacer sequence on crRNA.

Subsequently, the catalytic domains in Cas9 cleave the chromosomal DNA into a DSB, leading to the activation of the cellular endogenous repair with Non-Homologous End-Joining (NHEJ) and Homologous Directed Repair (HDR) [3] (Figure 1). Compared with previous genome editing methods (ZFN and TALEN), CRISPR/Cas9 is easy to operate and is programmable by simply changing the spacer sequence in the sgRNA. Therefore, CRISPR/Cas9 has replaced ZFN and TALEN and becomes the dominant system for genome editing [6].

CRISPR-Cas9 technology has dramatically facilitated precise genome-targeted manipulation and has been extensively applied to surgical filed in a wide variety of ways. However, numerous problems are concerned about fitness of edited cells, genome editing efficacy, various delivery methods such as virus, plasmid, mRNA, and nanoparticles, physical and chemical methods including electroporation, microinjections and lipid-mediated transfection, and potential off-target effects [7]. In particular, potential off-target effects should not be ignored. To apply CRISPR-Cas9 *in vivo* safely, endonuclease-induced off-target events should be minimized because indel formation at unintended loci may affect cell viability or instead promote tumorigenesis. Intensive research has been conducted to limit off-target events and improve the specificity [8]. Possible methods for reducing the risk of Cas9-mediated off-target include the use of paired Cas9 nickases, truncated gRNAs with shorter protospacer complementary regions, and high-fidelity Cas9 endonucleases.

Modification of the Cas9 protein to alter PAM preferences or enhance target DNA recognition can also be used to decrease off-target effects and thus enhance the on-target specificity [9]. Moreover, a synthetic switch was built to self-regulate Cas9 expression in both the transcription and the translation steps. The synthetic switch could simultaneously inhibit transcription and translation, thus rapidly attenuating the Cas9 expression. The restricted Cas9 expression minimized the off- target effects while increasing high efficiency and on-target indel mutation. Also, the synthetic switch can be integrated into viral vectors for self-regulating Cas9 expression, which provides a new "hit and run" strategy for *in vivo* genome editing [10].

At present, potential applications of CRISPR-mediated genome editing to surgical filed include cancer therapy and organ transplantation/regeneration, cardiovascular surgery, and neurological surgery. In this review, we present the potential applications of CRISPR/Cas9-mediated genome editing in the field of surgery.

Application of CRISPR/Cas9 Genome Editing to Cancer Therapy

Application potentials of CRISPR/Cas9 genome editing in cancer therapy are divided to 6 categories:

1) Genome editing for cancer cells themselves.

2) Genome editing for carcinogenic virus.

3) Genome editing for stromal cells.

4) Application of genome editing to anticancer drug development.

5) Genome editing for cancer immunotherapy.

6). Genome editing for oncolytic viral therapy [7].

Genome/epigenome editing for cancer cells themselves

Knockout of genes involved in the proliferation and survival of cancer cells remarkably reduces cancer cell growth and promotes apoptosis, thereby inhibiting tumor growth. Correcting the oncogenic genome/epigenome aberrations through CRISPR-Cas9 might represent a promising therapeutic strategy against cancer. In a cellular model of bladder cancer, for example, the expression of sgRNA and Cas9 was controlled by a cancer-specific hTERT (Human Telomerase Reverse Transcriptase) promoter and an urothelium specific hUP II (human Uroplakin II) promoter, respectively. Based on this method, Cas9 and sgRNA were co-expressed only in bladder cancer cells to activate suppressors such as p21, E-cad and h Bax [11]. In addition, Li et al. [12] recently exploited CRISPR interference and programmable base editing to determine their potential in editing a TERT gene promoter-activating mutation, which occurs in many diverse cancer types, particularly glioblastoma. Aubrey et al. [13] showed that knockout of MCL-1 gene in human Burkitt Lymphoma (BL) cells induced the apoptosis of BL cells at a higher frequency through the lentiviral CRISPR- Cas9 system. Similarly, with the CRISPR technology, the correction of PKC mutation reduced tumor growth in a xenograft model [14]. Interestingly, knock-in of a suicide gene HSV1-tk *via* Cas9 into the chromosomal breakpoints of the fusion genes caused cell death and decreased tumor size in human prostate and liver cancer models [15]. Alternatively, through CRISPR-Cas9-mediated homologous recombination, knock-in of a therapeutic transgene at a desired location induces cancer cell death.

Genome editing for carcinogenic virus

Utilizing the viral genome-specific Cas9-sgRNA, viral oncogenes can be directly targeted and eliminated as well as the genes required for viral maintenance and replication. The viral genome- specific Cas9-sgRNA suppress viral oncogene expression and ultimately induce cancer cell death.

Carcinogenic virus infection is a critical factor in the occurrence of cancer, 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. Cervical cancer is caused mainly by HPV. The expression of HPV oncoproteins E6 and E7 induces normal cells to undergo malignant transformation and then maintain malignancy [16,32,33]. Some studies have confirmed that targeting the E6 and E7 genes and their promoters with the CRISPR-Cas9 achieved the inhibition of cervical cancer growth and reversal of the malignant phenotype [17,34,35]. CRISPR-Cas9-mediated HBV DNA editing and deletion can effectively inhibit virus production in both cells and mouse models [36-39]. Also, CRISPR-Cas9 inhibits HBV replication thorough targeting of HBV antigen-encoded regions [18,40,41].

Genome editing for stromal cells

CRISPR-Cas9 can be used for the reprogramming of tumor stroma to achieve anticancer effects. Cancer cells and stromal cells such as fibroblasts form a dynamic and symbiotic relationship between cancer progression and treatment resistance. CRISPR-Cas9 can be used for the reprogramming of tumor stroma to achieve anticancer effects. Yang et al. [19] found that genes involved in glutamine synthesis were overexpressed in Cancer Associated Fibroblasts (CAFs) and. inactivation of Glutamine Synthetase (GS) effectively inhibits the growth of cancer cells, while inactivation of GS in cancer cells does not affect the ability of CAFs. Thus, CRISPR-mediated matrix GS knockout may be cost-effective in inhibiting tumor growth and achieving anticancer effects.

Similarly, Sherman et al. [20] revealed that a key regulator of pancreatic stellate cells is the Vitamin D Receptor (VDR). In a tumor model, VDR activation caused the reprogramming of reactive stroma and reduced fibrosis- associated inflammatory markers. Thus, the use of CRISPR/Cas9 technology can enhance VDR activation and effective drug penetration thorough stroma reprogramming. Compared with cancer cells whose genetic/epigenomic heterogeneity and dynamics are hard to follow, stromal cells are easier to genetically manipulate. Moreover, genetically edited stromal cells may not suffer from fitness disadvantage as therapeutically edited cancer cells.

Application of genome editing to anticancer drug development

With CRISPR-Cas9 technology, resistance genes are identified and the new drug genomic loci in cancer is discovered. Kasap et al. [21] utilized CRISPR-Cas9 to generate the A133P mutation in HeLa cells, which resulted in increased resistance to ispinesib in mutated cancer cells. It was also validated that kinesin-5 in cancer cells was a direct target of ispinesib. Similarly, CRISPR-Cas9-mediated SMARCB1 gene knockdown conferred resistance to the chemotherapeutic drug doxorubicin, indicating that SMARCB1 was a drug resistance-related gene [22]. The CRISPR-Cas9 knockout library containing 64,751 unique sgRNAs, was implemented in melanoma cells to seek new and more plausible candidate genes whose deletion conferred resistance to vemurafenib, a BRAF protein kinase inhibitor [23]. Also, CRISPR/Cas9 technology was applied to screen, and KAT2A was selected as a candidate for downstream research and the inhibition of KAT2A was proposed as a therapeutic strategy in AML [24].

Genome editing for cancer immunotherapy

The knockdown of these inhibitory receptor genes including PD-1 gene and CTLA-4 gene, using CRISPR-Cas9 technology, may be crucial to improve the efficacy of cancer immunotherapy. CRISPR-Cas9 has recently been used in the genome editing of primary human T cells in which PD-1 expression were reduced by CRISPR-mediated indel mutations [25,61]. Also, Su et al. [26,62] showed that precise knockout of the PD-1 gene remarkably decreased PD-1 expression via electroporation of plasmid-encoded sgRNA and Cas9 into human T cells. Clinical trials about CRISPR- mediated PD-1 gene knockout have been carried out in China for cancer treatment, such as castration resistant prostate cancer, muscle-invasive bladder cancer, and metastatic renal cell carcinoma, aiming to further evaluate the efficacy and safety of PD-1 knockout in T cells, for Chimeric Antigen Receptor T (CAR-T) cell therapy. CRISPR-Cas9 system can also disrupt multiple genomic sites simultaneously and yield universal CAR-T cells that are deficient in endogenous T Cell Receptor (TCR), HLA class I (HLA-I) and PD-1 [27].

Genome editing for oncolytic virotherapy

Some viruses can be genetically engineered to efficiently replicate within the host though CRISPR/Cas9 system. Thus, those viruses specifically infect and kill cancer cells, and subsequently induce anticancer immune responses. Talimogene laherparepvec (T-VEC), derived from Herpes Simplex Virus type 1 (HSV-1), is the first FDA-approved oncolytic viral therapy against advanced melanoma. It can specifically target cancer cells and promote Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) production to enhance anticancer immunity [28]. Replacement of Thymidine Kinase (TK) gene with Ret Finger Protein (RFP) gene *via* CRISPR-Cas9 in vaccine virus mostly leaded to RFP positive plaques. Actually, the CRISPR-Cas9 system also efficiently produced virus with the deletion of N1L and A46R [29].

Application of CRISPR/Cas9 genome editing to organ transplantation/regeneration

Genetically modified pigs are promising donors for xenotransplantation as they show many anatomical and physiological similarities to humans. Recently, most of the advances that have been made in the field of xenotransplantation because of the production of CRISPR/Cas9-mediated genome editing pigs. As a result, a large variety of CRIPSR-mediated genome editing pigs have been generated, and the production of source pigs with multiple edited genes has become easier and faster [30,92]. However, immunological rejection including Hyperacute Rejection (HAR), Acute Humoral Xenograft Rejection (AHXR), immune cell-mediated rejection, and other barriers associated with xenotransplantation must be overcome with various strategies for the genetic modification of pigs. Using CRISPR/Cas9 genome editing technology, it is now possible to easily knockout several porcine genes associated with the expression of sugar residues, antigens for (naturally) existing antibodies in humans, including GGTA1, CMAH, and β4GalNT2, and thereby preventing the antigen-antibody response. Moreover, insertion of human complement and coagulation regulatory transgenes, such as CD46, CD55, CD59, and hTBM, can further overcome effects of the humoral immune response and coagulation dysfunction, while expression of regulatory factors of immune responses can inhibit the adaptive immune rejection [31].

In addition to the concerns about pig-to-human immunological compatibility, the risk of cross-species transmission of Porcine Endogenous Retroviruses (PERVs) has impeded the clinical application of this approach. Using CRISPR-Cas9, Niu et al. [32] inactivated all of the PERVs in a porcine primary cell line and generated PERV-inactivated pigs *via* somatic cell nuclear transfer.

Their study highlights the value of PERV inactivation to prevent

cross-species viral transmission and demonstrates the successful production of PERV-inactivated animals to address the safety concern in clinical xenotransplantation.

Moreover, combination of CRISPR/Cas9 and iPSCs technologies can provide human organs from chimaeric pigs. iPSC derived from the patient who needs an organ could be injected into a genetically-modified pig embryo, enabling a human organ to develop which can subsequently be transplanted into the patient [33]. However, two questions still remain in this field. First, the currently-available human iPSCs do not develop chimeras when injected into blastocysts. It will be necessary to generate naïve human iPSCs to develop human/pig chimeras [34]. Second, safety and ethical issues remain in respect to developing human/pig chimeras. Human iPSC-derived chimeras would possibly human neural and germ cells, which evokes ethical controversy. However, with the advantage of CRISPR/Cas9, forced expression of specific genes can be used to guide human iPSC to target organs after blastocyst injection. This method has been successfully used to generate functional pancreas in pancreatogenesis-disabled Pdx1- knockout mice [35].

Application of CRISPR/Cas9 genome editing to cardiovascular surgery

The potential application of genome editing techniques in the cardiovascular field is still in the early stages of development. One method is to produce iPSCs from a patient and to perform *ex vivo* genome editing. After editing the iPSCs, the cells were differentiated into the desired type of cells and transplanted back into the patient. The other method is in vivo genome editing by directly targeting the gene of interest in the host organ [36]. With the implementation of Homology-Independent Targeted Integration (HITI), precise genome editing is even possible in non-dividing cells such as cardiomyocytes. HDR only operates in dividing cells, but this repair mechanism is not elucidated in the mainly post-mitotic mature cardiomyocytes besides cardiomyocytes, endothelial cells, smooth muscle cells, or cardiac progenitor cells, which are able to develop into all cardiac lineages, would be desired cell types for cardiac regeneration Precise *in vivo* genome editing in cardiovascular disorders is challenging due to the nonoccurrence of HDR in non-diving cells such as mature cardiomyocytes. The investigators developed a new genome editing tool based on CRISPR/Cas9, which makes use of the NHEJ repair mechanism: Homology-Independent Targeted Integration (HITI). Recently, Suzuki et al. [37] proposed a method enabling a specific modification of the endogenous sequence, even in non-dividing cells. They were able to show that the genome editing efficiency *via.* HITI is approximately ten times higher than HDR. This innovative method allows tailored *in vivo* genome editing in cardiomyocytes. Recently Gedicke-Hornung et al. [38] were able to recover the function of the mutated MYBPC3 gene encoding cardiac myosin-binding protein C by exon skipping *via* RNA modulation using AONs. NHEJ-mediated exon skipping was performed in Duchenne muscular dystrophy. Three separate groups published their work on exon skipping of the mutant dystrophin exon in neonatal or adult mdx (X chromosome-linked muscular dystrophy) mice [39-41]. The investigators utilized the CRISPR/Cas9 system to skip exon 23 and partially restored the dystrophin protein function. The Cas9 and sgRNA vectors were delivered by adeno-associated virus by intraperitoneal injection. Ding et al. [42] were able to prove this concept in their study about proprotein convertase subtilisin/kexin type 9 (PCSK9).

Application of CRISPR/Cas9 genome editing to neurological surgery neurodegenerative disease

Recent advancements in the field of CRISPR/Cas9-mediated genome editing offer a new template for dissecting the precise molecular pathways underlying the complex neurodegenerative disorders. Neuroinflammation, which is a hallmark of various neurodegenerative disorders, plays a critical role in the development and progression of these neurodegenerative diseases [43]. Activation of astrocytes and microglia induces the expression of proinflammatory cytokines and chemokines including GMF, IL1-β, IL-6, IL-8, TNF, IL- 12, IL-23, IL-33, CXCL10 and CXCL12. In neuroinflammation, there is increased phosphorylation of p38MAPK/ERK pathways, which leads to activation, and nuclear translocation of NFκB thereby causing increased oxidative stress, mitochondrial dysfunction and apoptosis [44]. CRISPR/Cas9-mediated genome editing is a powerful tool for inducing gene correction, disease modeling, transcriptional regulation, epigenome engineering, chromatin visualization as well as development of neurotherapeutics. Thus, the latest CRISPR-mediated gene editing approaches have been used to target various neurodegenerative diseases including Alzheimer's Disease (AD), Parkinson's Disease (PD), Amyotrophic Lateral Sclerosis (ALS), Frontotemporal Dementia (FTD), Multiple Sclerosis (MS), Spinocerebellar Ataxia (SCA), and Tay-Sachs Disease (TS) [44]. Precision- targeted genome editing of the key signaling molecular mechanisms underlying neuroinflammation offers a novel therapeutic approach to effectively treat neurodegenerative disorders and significantly reduce the economic burden.

Traumatic Brain Injury and Spinal Cord Injury

Traumatic Brain Injury (TBI) and Spinal Cord Injury (SCI) are disorders of the central nervous system with several causes, being the most common motor vehicle collisions in the younger population, followed by accidental falls in the elderly population [1,2, 45]. Traumatic Brain Injury (TBI) or Spinal Cord Injury (SCI) causes temporary disabilities or permanent effects including neuropathic pain and spasticity. The damage often triggers the neuronal inflammatory process.

Long-lasting neuroinflammatory response leads to aggravation of the neurodegenerative processes after injury. Some possible therapeutic targets for TBI or SCI are these activated signaling pathways, and new perspectives for TBI or SCI treatment are based on novel technologies including CRISPR- mediated genome editing [46]. These signaling cascades include the mechanistic target of mTOR, PTEN, NF-κB, C3 (complement system protein) and p38 [47]. The disruption of this pathway remarkably increases neuronal apoptosis and brain/spinal cord ischemia or reperfusion injury followed by microglia activation and neuronal degeneration [48]. Glial Maturation Factor (GMF) leads to p38 activation [49]. These pathways regulate the production of pro- and anti-inflammatory cytokines in microglia, the macrophages of the Central Nervous System (CNS) [50].

Proposed targets of TBI or SCI using CRISPR-mediated genome editing are as follows: The knockdown of GMF in microglia, that could decrease p38 mitogen-activated protein kinase pathway signaling and might reduce microglial activation; the knockdown of C3 (complement system protein) in astrocytes, that could improve axonal regeneration and might facilitate motor function recovery; and

the knockdown of PTEN in neurons, that might promote enhance in neurite length [46].

In summary, gene-based therapies using the CRISPR-mediated technologies we presented here offer a novel and promising precision medicine approach for TRI or SCI treatment.

Ethical Concerns

CRISPR/Cas9-mediated genome editing technologies, because of their enormous popularity, are required to discuss the ethical implication of genome editing in clinical therapy. Indeed, CRISPR/Cas9 genome editing technology has been applied to improvement of animal breeding as well as plant breeding [51,52]. Moreover, some clinical applications of CRISPR/Cas9 genome editing to human disease have been reported [53]. The National Academies of Sciences prepared a statement on the use of human genome editing. The committee concluded that genome editing in basic science and future clinical use of the technologies in somatic cells are covered by the existing regulatory measures of gene therapy. The debate about germline modification on the other hand needs to be further addressed including the international scientific community as well as different perspectives from society. In their updated version from 2017, the committee concluded though that research on germ-line editing should continue, but clinical trials need to be evaluated with a strict risk and benefit consideration [54]. Consensus standards need to be developed and implemented.

Fully understanding the risks of germ-line editing can lead the way to ensure a safe use of genome editing and enable an open productive discussion among science and society, especially in the most controversial field of genome editing, human enhancement.

Conclusion

In summary, CRISPR/Cas9-mediated genome editing technology, as a powerful editing tool, has tremendous therapeutic potential for increasing surgical approach, albeit with some challenges. CRISPR/Cas9-mediated genome editing therapy will herald a new era in the field of surgery. In addition, future research for surgical field should enable engineers, biologist, and chemists to work with surgeons and researchers for obtaining the state-of-the-art understanding of the various properties of surgical field, together with genetic and biochemical properties. The continuous advances and innovations in CRISPR-Cas9 technology will increase safety and effectivity of therapeutic strategies and bring the promise to patients in the future.

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