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Review Article

CRISPR/Cas9-mediated genome editing: from basic research to gene therapy

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ABSTRACT

CRISPR/Cas9 mediated genome editing is one of the most significant molecular tools discovered to edit the desired genes. It has ushered in a new era of novel possibilities of gene therapy. CRISPR/Cas9 system was originally observed as a part of the adaptive immune system in bacteria. It later on was adapted to carry precise and targeted alterations to the DNA in human cells to be used for gene therapy to correct genetic disorders and treat various severe diseases associated with the genetic changes. Besides this, the CRISPR/Cas9 system has been employed in pharmacogenomics to develop new drugs based on the patient's genes, in modifying the organisms for research and even for diagnostic purposes in developing CRISPR based COVID-9 test. The recent approval of a CRISPR/Cas9 cellular gene therapy by FDA named "Casgevy" to treat sickle cell anemia is a testimonial to the potentials of CRISPR/Cas9 system in developing innovative gene therapies. This review details the mechanisms of CRISPR/Cas9 gene editing and its utilization in the ongoing clinical trials in the treatment of not only the monogenic disorders like sickle cell disease, thalassemia, and genetic blindness but also in treating multi-factorial diseases like cancers, cardiac diseases, diabetes, autoimmune diseases, viral infections such as human immunodeficiency virus (HIV) etc. An attempt has also been made to discuss the various limitations, challenges and ethical frameworks encompassing CRISPR/Cas9 based gene therapy in clinical settings.

Keywords: Gene therapy, CRISPR/Cas9, CAR-T cells, Endonucleases, Homology-directed repair, Non-homologous end joining, Gene knock-out, Gene knock-in, Clinical trials, Ethics

INTRODUCTION

Gene therapy is a revolutionary medical technique to treat various genetic disorders. The fundamental principle of gene therapy is to address the root cause of the diseases at the gene level by introducing, silencing, modifying or editing the gene of interest to mitigate the specific genetic disorder. Gene therapy, though so far has been in initial stages, mainly restricted to the labs, but recent advancements in various gene therapy techniques have enabled the biomedical scientists to carry out their clinical trials to correct the genetic disorders with promising results. Presently the realm of gene therapy is no more restricted to treat genetic disorders only but also extends to the treatment of cancers and certain viral infections.

Brief history of gene therapy and various gene editing tools

The first approved clinical trial for gene therapy was a viral vector based therapeutic trans-gene delivery in 1990 for a monogenic disease, adenosine deaminase-severe combined immunodeficiency (ADA-SCID). It made use of the *ex vivo* delivery of wild type adenosine deaminase gene to the autologous T–lymphocytes which were put back into the two young girls suffering from ADA-SCID. This procedure though demonstrated suboptimal results in one patient only, it nonetheless led to many successive but unsuccessful gene therapy trials using viral vectors.[1,2] Another gene therapy attempt to deliver non-mutated ornithine transcarbamylase (OTC) gene using recombinant

adenoviral vector to treat an 18-year-old patient suffering from a mild form of ornithine transcarbamylase (OTC) deficiency also remained unsuccessful.[3] The patient died after 4 days from a severe immune response leading to multi organ failure. There exist various studies where adverse effects occurred in other gene therapy trials too including one in which *ex vivo* delivery of therapeutic transgenes, to $CD34^+$ hematopoetic stem cells infused to the patients for treating SCID, developed therapy associated leukemia. The leukemia was linked to the integration of the therapeutic gene into a specific lim domain only2 (LMO2 plays a crucial role in hematopoietic development) gene locus highlighting the risks of insertional mutagenesis with retroviral vectors.[4-6]

The above studies collectively warned about the safety of gene therapy trials in humans and consequently raised concerns to look out for new modalities for gene therapy. This led to the development of the re-engineered viruses, non-viral vectors as well as discovery of novel methods to directly edit the existing genetic mutation *in situ* to minimize insertional mutagenesis and adverse immune responses.[7] Gene therapy using non-viral vectors for gene delivery such as liposomes, inorganic particles and chemical based nanosized natural or synthetic polymers demonstrated less immunogenicity and better tolerance compared to the viral vectors as seen in certain cardiovascular clinical trials. However, these vectors failed to achieve the transfection effectiveness compared to viral vectors creating a major hurdle in their use in gene therapy.[8-10]

Endonucleases based new gene editing tools

The drawbacks and apprehensions associated with the viral and non-viral based vectors such as their delivery, interaction and penetration through cells, circulation time, immunogenicity, blood cancer risks, ability to transcript and maintain gene expression of the therapeutic gene etc. finally resulted in the discovery of alternative novel methods for gene therapy.

The more interesting alternative method was to directly edit the existing genetic mutation *in situ* rather than introducing the wild copy of the therapeutic gene. It was made possible by repurposing the enzymes called as nucleases to directly edit the existing genetic mutation. The varied types of advanced human gene editing technologies developed for gene therapy based on the engineered nucleases or bacterial nucleases have shown remarkable potential to alter the human gene of interest with minimal adverse effects. These are namely, transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), CRISPR/Cas system and homing endo nucleases or mega nucleases.[11-14]

What is CRISPR/Cas9 system

The CRISPR/Cas system in the last decade has come up as a very pioneering gene editing method due to its practical simplicity, highly precise, fastest and cost effective nature compared to the other existing gene manipulation technologies discussed above.[14] CRISPR is an acronym for clustered regularly interspaced short palindromic repeats and was first reported by Ishino et al from Osaka University, Japan in 1987 in the DNA sequences of the *Escherchia coli*. CRISPR/Cas9 of all the CRISPR/Cas (CRISPR associated protein) systems, is currently acclaimed as the most advanced and frequently employed method for genome editing in gene therapy.[14,15] CRISPR/Cas9 also fetched the Nobel Prize in Chemistry in the year 2020 to the two researchers, Emmanuelle Charpentier and Jennifer A. Doudna, for its discovery. The honour came within a period of less than a decade after the identification of key molecular components of the CRISPR/Cas9 system.[16] The CRISPR/Cas systems are commonly divided into two classes, class I and class II, on the basis of structural variation of the Cas genes and their organization form.[17] The most regularly used is the type II CRISPR/Cas9 system, for gene editing, which makes use of a single Cas protein from *Streptococcus pyogenes* (SpCas9) to target specific DNA sequences.[18]

CRISPR/Cas9 gene editing, called as "genetic scissors", has now revolutionized the clinical perspective for targeted interventions across the spectrum of varied genetic disorders. The very recent approval by the FDA for the use of CRISPR/Cas9 based cellular gene therapy, named "Casgevy" for treatment of sickle cell disease or transfusion dependent beta thalessemia (TDT) in patients 12 years of age and older with recurrent vaso-occlusive crises, affirms its practical potentials to treat genetic disorders.[19] Moreover, it eliminates certain concerns like immunogenic toxicity and insertional onco-genesis alarmed by the conventional methods of therapeutic gene therapy.[20]

STRUCTURE AND WORKING OF CRIPSR/CAS9 GENE EDITING TECHNOLOGY

CRISPR/CAS9 as defense system in bacteria

CRISPR/Cas system was originally observed as an adaptive defense mechanism in the prokaryotes mainly bacteria and archaea to protect them from mobile genetic elements (MGEs) particularly the bacteriophages, transposans or plasmids that have earlier infected these prokaryotes. These MGEs acquired by the bacteria during bacteriophage attack are integrated into the palindromic repeats of the bacterium genome as spacers which it retains as memory of past infections. The different spacers (variable sequences of viral genome) between the palindromic sequences together constitute a CRISPR array which on transcription form a crisprRNA (crRNA) (Figure 1).[21]

CRISPR associated nuclease9 (Cas9) protein was discovered as a single effector protein having nuclease activity in *Streptococcus thermophillus* and is encoded by a single large gene as compared to other Cas genes in

CRISPR/Cas systems. Cas9 is also the most characterized and extensively studied CRISPR associated nuclease. Cas9 has two DNA cleavage domains which slip into either stands of DNA to cut the desired double stranded DNA. The other molecular component in the CRISPR/Cas system is called trans-activating CRISPR RNAs (tracrRNA) which are complementary to and can anneal the palindromic sequences in crRNA (Figure 2).[21,22]

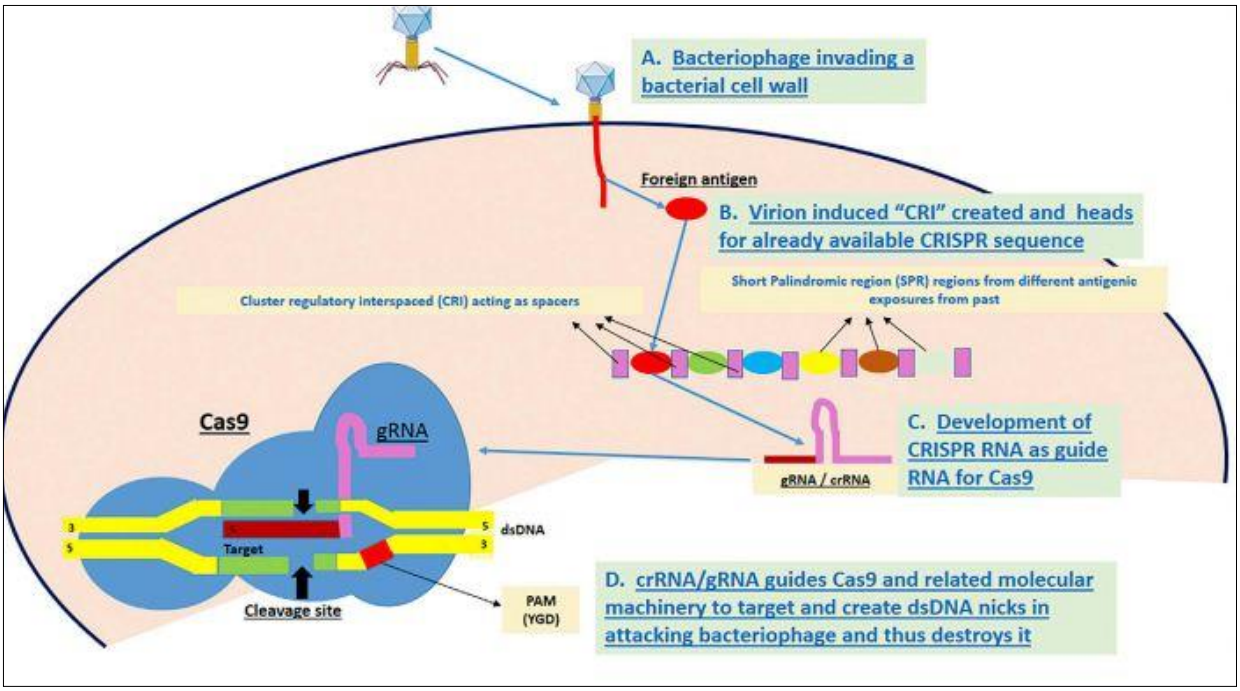

Figure 1: Graphic representation: mechanism of how CRISPR/Cas9 targets and destroys bacteriophage genome at a specific splicing site using crRNA/gRNA.[87]

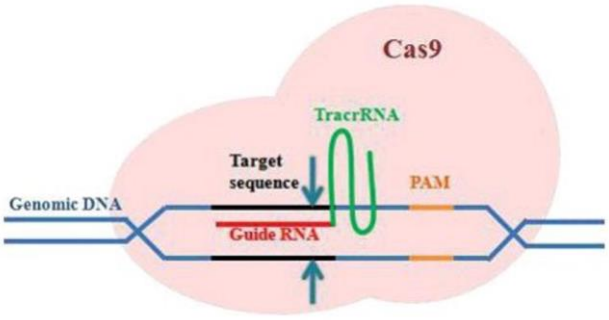

Figure 2: The components of the CRISPR/Cas9 system: Cas9, PAM and guideRNA=CRISPR-RNA(crRNA) + trans-activating crRNA (tracrRNA).[88]

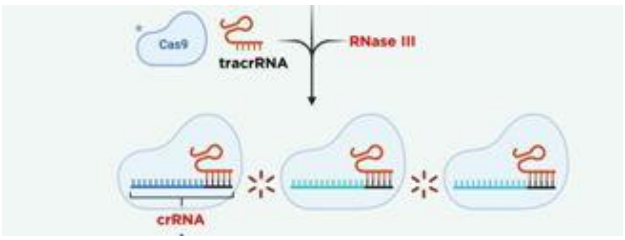

Figure 3: The 'effector crRNA' complex=crRNA + tracrRNAs + the associated Cas9 enzyme.

The crRNA, tracrRNAs and the associated Cas9 enzyme form a single complex which on cleavage by double stranded endonuclease, namely ribonuclease III (RNAse III), yields individual 'effector crRNA' complexes (Figure 3). This individual effector crRNA complex in a bacterium immediately recognizes, if any, the foreign (invading) viral genome having sequence complementary to its crRNA. This, in turn, is cut by the Cas9 in the effector complex at a site few base pairs upstream of a 5-6 nucleotides unique viral sequence, known as protospacer adjacent motif (PAM). The whole process finally results in viral neutralization as its genome can no longer be transcribed, further enabling bacteria to use it as a defense mechanism against the bacteriophage (Figure 1).[23]

Working mechanism of CRISPR/Cas9 system as a gene editing tool

The CRISPR/Cas9 system for adaptive defense immunity by the bacterium discussed above was repurposed for genome editing in humans by joining the crRNA and the tracrRNA (which are separate molecular entities in a bacterium) by a linker to form one piece of RNA called guide RNA (gRNA). The guide RNA (gRNA) contains the specific sequence of 18-20 nts that matches the gene target to be edited in the genome. The gRNA, when put into

human cells, navigates or directs Cas9 sourced from *Streptococcus pyrogenes* to the precise location (matching sequence) in the genome to cut the DNA upstream of the appropriate PAM sequence and produce the double stranded DNA breaks (DSBs).[24] The gRNA, thus, acts as a molecular "GPS" that navigates the Cas9 enzyme to specific location on the DNA where the gene editing is required (Figure 4). This complete mechanism acts in a similar way as the three components of the system perform conjointly in bacteria. The guide RNA can be made in the lab as a single strand synthetic single guide RNA (sgRNA). The targeted site in the sgRNA must precede the specific PAM sequence necessary for the compatibility of the particular Cas9 to cut the DNA and generate the double stranded breaks. By doing so, CRISPR/Cas9 system is known to edit DNA theoretically as specific as single base pair (Figure 4).[25]

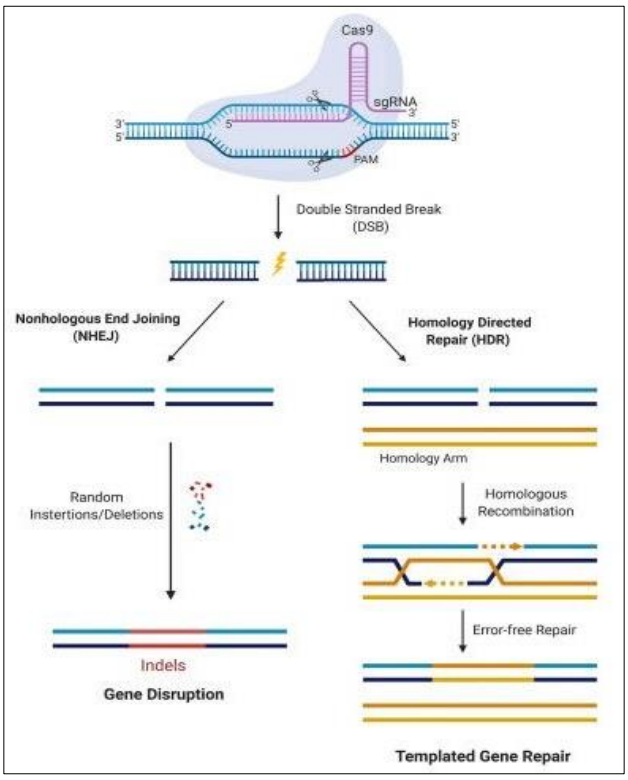

Figure 4: CRISPR/Cas9 methodology of action: sgRNA recognizes the target sequence followed by CAS9 endonuclease activity creating double stranded DNA cleavage downstream of PAM. DSBs are later on repaired via NHEJ or HR repair mechanism.[89]

Homologous and non-homologous recombination

The DSBs formed due to Cas9 incision are later on joined together either by homology directed repair (HDR) or non-homology end joining (NHEJ). HDR requires a template DNA strand together with the CRISPR/Cas9 complex to repair the broken DNA. The template enables the precise incorporation of particular alterations or modifications at the target site. HDR is a high fidelity repair pathway as it lowers the probability of errors in the editing process due to absence of indels i.e. insertions or deletions of DNA sequences and maintains the uniformity in the size of DNA.[26] NHEJ involves direct ligation of the dsDNA breaks without the requirement of a homologous template which may result in indels at the joining ends leading to non-uniformity in edited DNA strand. NHEJ repair, thus may lead to gene mutations (Figure 4).[27,28]

How to get CRISPR/Cas9 into the targeted cells

The three most commonly used approaches of transfer of CRISPR/Cas9 into the targeted cells for achieving genome editing are: the plasmid-based method, which requires integrating both the Cas9 gene and sgRNA into a single plasmid for sustained expression, despite the challenge of getting the plasmid into the cell nucleus; directly delivering Cas9 mRNA and sgRNA into cells, a technique restricted by mRNA's higher rate of instability and ensuing temporary gene modification only; introducing Cas9 protein and sgRNA directly, providing fast, stable, and less antigenic method of gene editing.[29-31] The CRISPR/Cas9 has additional potential of simultaneously editing multiple loci making this technology easier, efficient, and expandable in comparison to other genome editing tools like TALENs, and ZFENs.[32] The precisely altered targeted genes by CRISPR/Cas9 are finally used either to correct genetic disorders, develop genetically modified organisms, and advancing various other biomedical and clinical applications such as in drug development and pharmacogenomics.[33-35] CRISPR/Cas9 technology has even been manipulated for diagnostic purpose as demonstrated by the development of CRISPR based COVID 19 test which is known for its accuracy and rapidity.[36]

APPLICATIONS OF CRISPR/CAS9 GENE EDITING TECHNOLOGY IN GENE THERAPY

The use of CRISPR/Cas9 gene editing technology has generated significant interest in the field of gene therapy because of its promising potential for precise and targeted modifications of the human genome for therapeutic purposes. The delivery of CRISPR gene therapy for therapeutic purposes is done either *in vivo* or *ex vivo* (Figure 5). There exists multiple studies on CRISPR/Cas9 gene editing and a few of these are in clinical trials to investigate the viability of using CRISPR/Cas9 gene therapy to treat specific genetic mutations especially in blood disorders e.g. sickle cell anemia, and cystic fibrosis; target and modify onco-genes; modify T cells to enhance immunotherapy (also known as chimeric antigen receptor T cell therapy, and CAR-T cell therapy) particularly in hematological malignancies (Figure 6); in infectious diseases such as disrupting the HIV and hepatitis B virus from replicating; in neurological disorders to modify the mutated gene responsible for Huntigton's disease; and to edit the gene for an inherited eye disorder responsible for childhood blindness-Leber congenital amaurosis.[37-42] The CRISPR/Cas9 is making advances in precision medicine in customizing the therapy based on the genetic make-up of an individual which is discussed in the following sections.

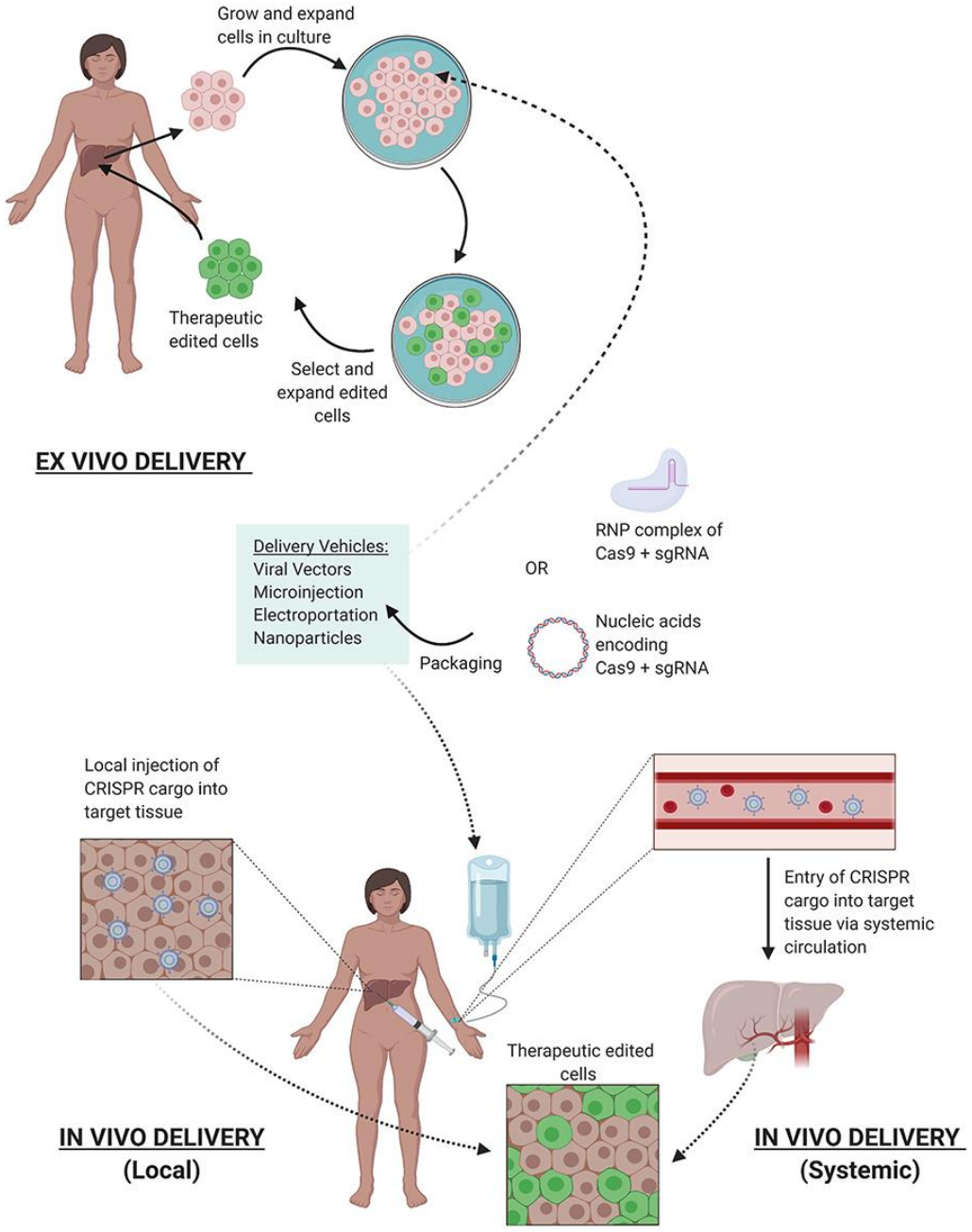

Figure 5: Delivery of CRISPR therapy: nucleic acids encoding CRISPR/Cas9 or its RNP complex can be packaged into delivery vehicles. Once packaged, edits can be facilitated either ex vivo or in vivo. Ex vivo editing involves extraction of target cells from the patient, culturing those cells for their expansion in vitro, delivery of the CRISPR components to yield the desired edits, selection, and expansion of edited cells, and finally reintroduction of therapeutic edited cells into the patient. In vivo editing can be systemically delivered via intravenous infusions to the patient, where the CRISPR cargo travels through the bloodstream via arteries leading to the target tissue, or locally delivered with injections directly to target tissue for therapeutic benefits.[90]

Blood disorders

The genetic disorders of the blood mainly sickle cell disease (SCD) and transfusion-dependent beta thalassemia (TBT) affecting hemoglobin in RBCs were early targets for CRISPR-based gene editing. The focus during ongoing clinical trials is on activating the gene for fetal hemoglobin (HbF), specifically present in fetuses and young infants only. The activation of the HbF back in SCD or TBT patients compensate for the defective hemoglobin seen in these patients. Vertex Pharmaceuticals® along with its co-developer CRISPR Therapeutics® in their clinical trials (NCT03655678 for CLIMB THAL-111 for thalassemia and NCT03745287 for CLIMB SCD-121 for sickle cell disease) electroporated $CD34^{+}$ hematopoietic and progenitor cells from healthy individuals with CRISPR/Cas9 to target the erythroid specific enhancers of

BCL11A.[43] This resulted in the modification of 80% of the alleles at this locus with no case of off target editing. The BCL11A is a transcription factor that inhibits Y-globin and fetal hemoglobin expression in erythroid cells. The two patients one each of TBT and SCD after myeloablation were infused with autologous $CD34^+$ cells edited with CRISPR/Cas9 targeted to the same BCL11A enhancer. Both patients showed high levels of allelic editing in bone marrow and blood, increased fetal hemoglobin that were distributed pan-cellularly, transfusion independence, and elimination of vaso-occlusive episodes in patient with SCD.[44,45]

The 95% of the TBT patients in phase 3 clinical trials who were monitored for three years by Vertex Pharmaceuticals® and CRISPR Therapeutics®, no longer required transfusion following the treatment. All of the participants with SCD following treatment were free of the vaso-occlusive crises that characterize the illness.[46] This CRISPR based therapy appears to be an effective treatment cure for SCD and TBT even without directly repairing the mutations that cause these disorders. The CRISPR/Cas9 based cellular gene therapy named "Casgevy" by The Vortex Pharmaceuticals® and CRISPR Therapeutics® have very recently been given approval by the US FDA and European Commission for treatment of patients of sickle cell disease or transfusion dependent beta thalessemia who are eligible for stem cell transplant but don't have an available donor.[47]

Cancers

The final aim of CRISPR/Cas9 in cancer therapy is to get rid of the mutations and restore the normal DNA bases in cancerous cells. PACT Pharma Inc., USA, recently developed CRISPR engineered T cells based on the tumor genome of sixteen individuals with metastasized head and neck, colorectal, bladder, ovarian, prostate, breast and lung cancer.[48] The gene edited T cells reprogrammed using CRISPR, by simultaneously knocking - out endogenous and knocking-in patient specific T cell receptors (TCRs) genes, enable the T cells to target the mutations specific to each patient's tumor. These clinical grade engineered T cells having neoantigen-specific TCR (neoTCR) which on infusion predominantly infiltrated tumors with reduction of tumor size in one of the treated individuals in the US based phase 1 trial (NCT03970382). This study illustrated the viability of non- viral precision gene editing using CRISPR to develop neoantigenic specific transgenic T cells having potential to traffic the tumors as confirmed by the presence of neoTCR in post infusion tumor TCR biopsy.[49]

US based Caribou Biosciences conducted phase 1 multicentre trial named "ANTLER" of single infusion of CB-010 on patients with aggressive non-Hodgkin's lymphomas. CB-010 is an allogenic CRISPR based anti-CD19 CAR-T cell therapy (Figure 6) with a second genetic modification of PD1 gene knockout which has shown a complete remission with acceptable safety and tolerance. PD1 gene is used by cancerous cells to evade the immune system. The US FDA based on the initial data has granted CB-010 therapeutic product, a regenerative medicine advanced therapy (RMAT) and fast track designation.[50]

The CRISPR/Cas9 gene editing technology is also being exploited for generating specific cancer models, opening the doors for research in functional cancer genomics and new cancer therapies. An attempt has been made to develop the organoid cancer models *in vitro* with the use of CRISPR gene editing tool to introduce mutations of tumor suppressor genes such as APC, TP53, SMDA4 etc. and modification of onco-genes like KRAS and PI3K.[51] The CRISPR-engineered tumor organoids in mice have been developed by delivering viral vectors, through mucosal injections guided by colonoscopy, containing CRISPR/Cas9 components to the distal colon to study tumor progression through an adenoma-carcinoma-metastasis sequence.[52]

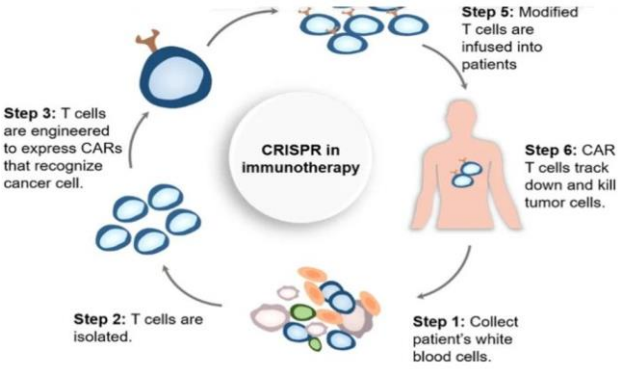

Figure 6: Diagrammatic representation of production of chimeric antigen receptor–T cells (CAR-T cells) with CRISPR genome editing technique: In CAR-T cells, the synthetic receptors consisting of antigen recognition, signaling, and co-stimulatory domains are utilized to reprogram T-cells employing CRISPR/Cas9 system to target tumor cells for destruction. Hence, the name CAR-T cell therapy.[91]

Cardiovascular diseases

The studies on the CRISPR/Cas9 based cardiovascular diseases prevention by knocking out diseases causing particular genes or repairing mutations such as hERG gene (codes for alpha subunit of a potassium ion channel) can be effective in treating long QT syndrome (LQTS). This is an autosomal dominant congenital heart disease known for sudden death due to ventricular arrhythmia.[53] The studies are also in progress in hepatocytes using CRISPR Cas 9 editing to generate nonsense mutations to APOC3 (apolipoprotein C3) or PCSK9 (proprotein convertase subtilisin/kexin type 5) genes to inhibit their proteins synthesis which are consequently known to lower significantly the blood LDL-cholesterol and triglycerides levels.[54,55] This may prove to be a long lasting therapeutic

modality compared the other drug inhibitors for such lipid disorders.

Hereditary transthyretin amyloidosis known as hATTR amyloisosis is a life threatening disease due to the progressive accumulation of mis-folded protein transthyretin (TTR) mainly in the cardiac tissue and nerves. NTLA-2001, a gene editing therapeutic agent to treat amyloidosis, developed by Intellia Therapeutics, Massachusetts, USA, uses CRISPR/Cas9–based *in vivo* gene-editing for the targeted knock out of TTR in patient's hepatocytes which are the main site of the production of mis-folded TTR. NTLA2001 is administered intravenously in a lipid nanoparticles (LNP) which finally gets accumulated in the hepatocytes. A single dose of NTLA -2001 markedly decreased (85%) the level of mutant TTR with a significant parallel decrease in symptoms of cardiomyopathy and neuropathy within an ongoing phase 1 study and has been granted approval for the phase 3 trials by the US FDA.[57]

Diabetes

The human induced progenitor stem cells (iPSCs) isolated from the single- gene diabetic MODY (maturity- onset diabetes of the young) patients have been gene edited for the particular diabetes related mutated gene like HNF4A, PDX-1, INS and GCK by CRISPR/Cas9 technology. The edited iPSCs on differentiation into pancreatic progenitor cells are transplanted into diabetic MODY patients for therapeutic purposes.[56] The allogenic pancreatic endoderm cells having immune associated genes genetically modified by CRISPR/Cas9 were transplanted in type-1 diabetic (T1-D) patients to eliminate the need of using immune-suppressants. These studies are still underway in phase I/II trial (NCT05565248) by CRISPR therapeutics AG (South Boston, MA, USA) in collaboration with Viacyte (San Diego, CA, USA).[57] The CRISPR/Cas9 genetically edited allogenic cells were implanted subcutaneously in T1-D patients in a protective device or pouch fitted with portals to allow vascularization around the pouch, bringing oxygen and metabolic nutrients from the blood to the cells, and taking up insulin from the cells. These cells also have two gene knockouts (B2M, TXNIP) and four insertions (PD-L1, HLA-E, TNFAIP3, and MANF) to improve functionality by reducing T- and NK-cell mediated immune rejection. The TNFAIP3 gene insert induced engraftment and protection against cytokine-induced apoptosis whereas the MANF gene insert enhanced β-cell proliferation and protection against inflammatory stress.[58]

Genetic blindness

Leber congenital amaurosis (LCA) is a mono- gene disorder resulting in childhood blindness because of a loss-of-function mutation in the CEP290 gene. A novel CRISPR/Cas9-based therapy, EDIT-101, uses AAV5 viral vector based delivery of the *Staphylococcus aureus* Cas9 and two guides targeting the ends of the CEP290 locus containing the IVS26 mutation directly into the retina.[59] The therapy specifically addresses the IVS26 intronic mutation in the CEP290 locus allowing for the production or translation of a functional protein. This *in vivo* CRISPR/Cas9 approach appears to be a significant advancement towards potentially curing genetic blindness, though its potential immune-toxicity and off-target effects (OTEs) need monitoring.[59]

Autoimmune diseases

The genes, the A20 deubiquitinase (DUB) and chromosome X open reading frame (CXorf21) have been targeted by CRISPR/Cas9 for treatment of systemic lupus erythematosus (SLE). The CRISPR/Cas9 knockdown of CXorf21 decreased the expression of TNF-alpha and IL-6. This study suggested the sexually dimorphic expression of CXorf21 in female immune cells as a risk factor for SLE.[60] Similarly, CRISPR/Cas9 generated U937 monocytes with A20 DUB -inactivating C103A knock in (KI) mutation up-regulated the levels of peptidyl arginine deiminases4 (PADI4), an enzyme involved in protein citrullination and neutrophill extracellular trap (NET) formation which consequently results in an increased susceptibility to SLE. It explained the contribution of A20 DUB-domain polymorphisms in the autoimmune pathogenesis of SLE and underpinned PAD4 as a target for the prevention and treatment of this disease.[61]

CRISPR Therapeutics® is initiating a trial on SLE in 2024, first of its kind in an autoimmune disorder with their next-generation CD19-targeting CAR-T cells using CTX112 agent. The latter is a next-generation, allogeneic CD 19 – directed autologus CAR -T product genetically modified ex vivo using CRISPR Cas 9 for long lasting remission.[62]

Viral and bacterial infections

In a recent study, CRISPR technology has been utilized for therapeutic benefits in COVID-19 using the therapeutic product, Cas13d. The latter is a RNA guided RNA targeting CRISPR endonuclease variant from *Ruminococcus flavefaciens*. CRISPR Cas13d has been harnessed to develop prophylactic antiviral CRISPR in human cells (PAC-MAN) to simultaneously degrade multiple RNA targets. It makes Cas13d, a broad spectrum antiviral strategy against SARS-CoV2 which is well known for its high mutations and recombination rates. The study designed and screened the CRISPR RNAs (crRNAs) targeting conserved viral regions and identified functional crRNAs targeting SARS-CoV-2. This advancement also highlights CRISPR's original role in combating viral infections in bacteria.[63]

Gene editing platforms have emerged recently as antiviral therapeutics for treating HIV by altering the host genes such as CCR5 (a key receptor in HIV infection) required by the virus or by targeting the viral genes necessary for replication.[64,65] Recently in 2022, the CRISPR based in *vivo* treatment, EBT-101, for the HIV has been dosed in

the first participants in US clinical trials by Excision Biotherapeutics, CA, USA. The treatment was infused by using AAV9 viral vector containing the gRNA which directs the Cas9 to excise the HIV genome in the cells at three distinct sites in the host genome thereby minimizing potential viral escape. The individuals on finding no adverse effect would be shifted to stage 2 trials by stopping the anti-retroviral therapy so as to check the efficacy of the CRISPR therapy as a cure.[66]

The US based Locus Biosciences has recently completed phase1b trial of LBP-ECO1, a CRISPR Cas3 treatment for urinary tract infection. It uses three engineered bacteriophages containing CRISPR Cas3 delivered directly to the bladder by catheter to target three main strains of *E. coli* responsible for urinary tract infection. Cas3 like Cas9 is also a CRISPR associated protein that acts as a 'molecular shredder' to degrade large segment of DNA. The initial result of this CRISPR enhanced bateriophage (crPhage) based therapy showed a significant decrease in the level of *E. coli* without affecting the commensal bacteria in the bladder of the participants.[67] This is the world's first precision medicine based completed, randomized, placebo-controlled trial of recombinant bacteriophage therapy and is ready for phase 2 human efficacy trial.[67,68]

Inflammatory disease

Hereditary angioedema (HA) which affects 1 in every 100,000 people is severe inflammatory diseases with attacks occurring every one to two weeks causing edema in the arms, legs, face, intestines and in the airway which can be life threatening without treatment. The dysregulation of the peptide bradykinin is the cause of edema which gets activated by plasma kallikrein, a serine protease. The kallikrein is therefore the therapeutic target and its inhibitors are the approved therapeutic agents. Intellia has recently developed NTLA-2002, a CRISPR/Cas9 based gene therapy to inactivate the gene that translates to plasma prekallikrein (KLKB1), a zymogen for kallikrein. It uses LNP to deliver in hepatocytes the mRNAs encoding the Cas9 endonucleases and a sgRNA to target KLKB1. It resulted in a dose dependent decrease in plasma kallikrein and on an average 95% decrease in monthly angioedema attacks. European Medical Agency (EMA) has designated NTLA-2002 as priority medicine (PRIME) and is ready for phase 3 trials in third quarter of the year 2024.[69]

LIMITATIONS AND CHALLENGES

The impact of CRISPR/Cas9 technology on gene therapy in clinical trials has been briefly discussed in this review. It is obvious from the studies presented here that CRISPR/Cas9 is a revolutionary gene-editing tool that has drastically changed the landscape of genetic research and has offered promising approach for treating genetic disorders and other diseases. However, its potential applications in clinics face serious obstacles which limit its overall effective translation to customized gene therapy. The main hurdles are its off target effects, delivery techniques, stimulation of counter-productive immune response besides the ethical concerns. However, the ongoing studies addressing these issues discussed in the following sections may bring forth novel possibilities to overcome the impediments in use of CRISPR effectively in the clinical space.

Off target effects

The off target DNA strand cleavages as a result of PAM and sgRNA mismatches lead to off target mutations and DNA stands rearrangement because of inversions and translocations. Such off targets may cause malignant tumors although probability of their occurrence is extremely low but are not acceptable in clinical settings.[70] The recent advancements in the PCR and next generation sequencing, availability of sophisticated software and novel unidirectional targeted sequencing (UDiTAS) have been put to use to accurately track such off target mutations.[71,72] The advances in base editing techniques such as base and prime editors and their use have also reduced the frequency of off target mutations and chromosomal rearrangements.[73,74]

Delivery mechanism

There exist substantial barriers in the safe and efficient transport of CRISPR gene editing components into human cells. The biggest challenge ahead lies in *in vivo* delivery of CRISPR/Cas9 system. The various current delivery methods like use of adeno- associated and lentivirus viral vectors, or non-viral methods such as lipid or polymer based nanoparticles and physical methods of microinjection/electroporation have serious limitations of their efficiency, potential for immune reaction or ability to target specific cells or tissues.[75-77] The technical challenge is to achieve high concentration of CRISPR/Cas9 components in all the target cells of the tissue to have a successful therapeutic use. The possible solutions include the rational designing of next generation materials for more effective delivery such as use of hybrid viral vectors, use of mini circle DNA which are more efficient and less immunogenic in place of plasmid DNA per mass, non-viral polymers conjugated to the gold nano-particle hybridization system etc.[78-80]

Immune response

The elicitation of immune response as an off target effect involves the production of antibody, facilitated through adaptive immune system, to Cas9 protein derived from *Streptococcus pyogenes*. Moreover, the presence of antibodies to Cas9 from *Streptococcus pyogenes* and *Staphylococcus aureus* have also been seen in a certain human population which implies pre-existing immunity to Cas9 due to prior exposure. This raises not only the risk of immediate rejection of the components of CRISPR/Cas9 but also generate memory cells to target Cas 9 during

subsequent therapies. Such an immune reaction can neutralize the CRISPR/Cas9 system before its intended function in the target cells.[81]

The viral vectors to deliver CRISPR/Cas9 components into the cells can be recognized as pathogens by the human immune system inducing inflammatory response via activation of innate immunity, cell death or dysfunction by T cell mediated cytotoxicity, thereby impairing the efficacy of the CRISPR therapy.[82] An attempt has been made to identify and alter immunogenic epitopes within the Cas9 protein to reduce its visibility to immune cells so as to mitigate the immune response.[83] The completely synthetic Cas9-like proteins or alternative Cas9 proteins from less immunogenic bacteria are also under investigations which might evade detection by the human immune system.[84] The immune response despite all these attempts to mitigate it, however still remains a prominent limitation to the safe and effective use of CRISPR/Cas9 in clinics.

Ethical concerns

The outcomes of the ongoing gene therapy trials using CRISPR in severe diseases such as malignancies and debilitating monogenic diseases will determine how fast the CRISPR system can be used to treat less severe diseases. The success of various CRISPR based gene edited human trials may also open a door for human genome editing by CRISPR for purposes other than the medical. One such biggest ethical concern about the CRISPR/Cas9 gene editing technology is its use for altering the genetic makeup of the embryos by the profit seekers for eugenic purposes i.e. to create offspring with certain preferred phenotypes or aesthetic traits. The irreversible changes in the germline cells by CRISPR/Cas9 tool will be passed on to the future generations which surely increases the ethical stakes significantly.[85] The apprehension about unnatural selection for unethical purposes has become more recognized in public perception because of the enormous media coverage surrounding the edited "CRISPR babies".[86] Keeping it in view, the regulatory bodies worldwide like FDA in USA and EMA in Europe are tasked with setting guidelines to balance such an innovation with ethical considerations and regulatory compliances. The need of the time is to have a call for an accurate, accountable and humanistic monitoring ensuring that CRISPR gene editing technology is used responsibly and for the maximum benefits of mankind.

CONCLUSION

CRISPR/Cas9 has emerged as one of the most seminal biotechnological innovations in recent years in the field of gene therapy. It has allowed biomedical researchers to edit precisely the DNA of the cells either by removal, addition or alteration of a section of DNA. The editing by CRISPR/Cas9 makes use of bacterial Cas9 enzyme as a pair of molecular scissors guided by a custom- designed RNA sequence to precisely cut the DNA of choice to cure the genetic disorders by rectifying the mutation at its source directly. CRISPR/Cas9 is more specific and efficient than earlier forms of gene editing and is therefore more suitable for gene therapy whose sole aim is to correct the defective or mutant genes responsible for development of diseases. This, in turn, has laid the foundation for revolutionary advancements in the field of medicine.

The outcome of the ongoing clinical trials using CRISPR technology offers a ray of hope even for the cure of complex multi-factorial diseases like heart diseases, cancer, neuro-degenerative disorders etc. as well. The very recent clinical trials using CRISPR/Cas9 to edit immune cells to target cancerous cells and HIV virus in infected cells have shown promising results. However, despite its profound potential benefits in gene therapy, the technology faces significant risks and ethical challenges in regard to possibility of off target effects and germline modifications for eugenics purposes. The attempts are already underway to address these issues. Future research directions are needed on improving the delivery mechanisms, enhancing editing precision, and resolving ethical and safety concerns. It is imperative that stringent ethical guidelines and policies are framed to ensure that CRISPR/Cas9 is only used for the betterment of human health. In conclusion, CRISPR/Cas9 has proved to be a revolutionary tool in the realm of gene therapy eliciting both hope and caution in equal measure.

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