Bioresearch Communications
Volume 06, Issue 01, January 2020
Journal Homepage: www.bioresearchcommunications.com

Review Article

Uphill Battle of CRISPR/Cas9: A Budding Technology in Need of Refinement

Gen Kaneko, Crystal Salinas, Hashimul Ehsan*

Department of Biology, University of Houston-Victoria, 3007, N. Ben Wilson, Victoria, TX 77901

ABSTRACT: CRISPR/Cas9 has been a discovery that has developed advancements in the field of genetic engineering. This technology revolutionized genome editing by its ease of manipulation, high efficiency and broad range of applications within a number of hosts and a variety of diseases. Scientists have invested a lot of their attention in this system to reap the beneficial consequences of genome manipulation in animal cells. CRISPR has potential that we have yet to consider or uncover, but this is just the steppingstone to something far more than a system of genomic modification. It is still in its infancy, and thus far, there has been many developments since its discovery. To date, CRISPR/Cas9 requires refinement to hone its accuracy of delivery and safety. Its application to many different diseases, ranging from abnormal cell growth to neurodegenerative disorders, reveals an amalgamation of results, both positive and negative in outcome. This reveals the climate surrounding CRISPR/Cas9 progress.

Keywords: CRISPR/Cas9, gene-editing technology, genomics, bioethics, media portrayal, regulations, enhancement, germline alterations

Article History
Received: 17 September 2019
Accepted: 21 December 2019

Scan the QR code to see the online version or,visit- www.bioresearchcommunications.com

Corresponding author
Dr. Hashimul Ehsan
Centre for Advanced Research in Department of Biology, University of Houston-Victoria, 3007
N. Ben Wilson Victoria, TX 77901
Email: ehsanh@uhv.edu
Phone: [361]570-4289

Citation: Hashimul Ehsan; Uphill Battle of CRISPR/Cas9: A Budding Technology . ***Biores Comm*.** 6(1). 859-871.

1. General information on CRISPR-Cas9 Genome Editing

Genome editing is a type of technology that utilizes modified nucleases engineered to manipulate genetic information in the genome of organisms. Introducing CRISPR/Cas9, geneticist have begun using nucleases as a way to genetically modify the eukaryotic genome with more successes than with previous methods [1]. CRISPRs are a class of repeated DNA sequences that associate with Cas proteins to play a vital role in bacterial and archaeal immunity against foreign viral nucleotides within a single cell [2]. There are three types of CRISPR/Cas systems that have been accepted by the scientific environment, Cas3 in type I systems, Cas9 in type II, and Cas10 in type III, but recently studies have demonstrated that possibility of a fourth class of CRISPR/Cas system may possess similarities to an ancestral innate immunity system that gained adaptive immunity [3]. Of the four possible systems, the type II CRISPR/Cas9 system has been the prime focus of most studies. Like type I, the type II system is known to target DNA. This system works by cleaving the invading viral DNA into small fragments called spacers, which are acquired and inserted into the CRISPR locus. The CRISPR system then expresses the Cas9 protein and begins to transcribe the CRISPR into pre-CRISPR RNA (pre-crRNA), synthesizing into CRISPR RNA or crRNA. The target nucleic acid is then recognized, targeted, and manipulated by association of the crRNA/Cas9, transactivating CRISPR RNA (tracrRNA) **(Figure 1).**

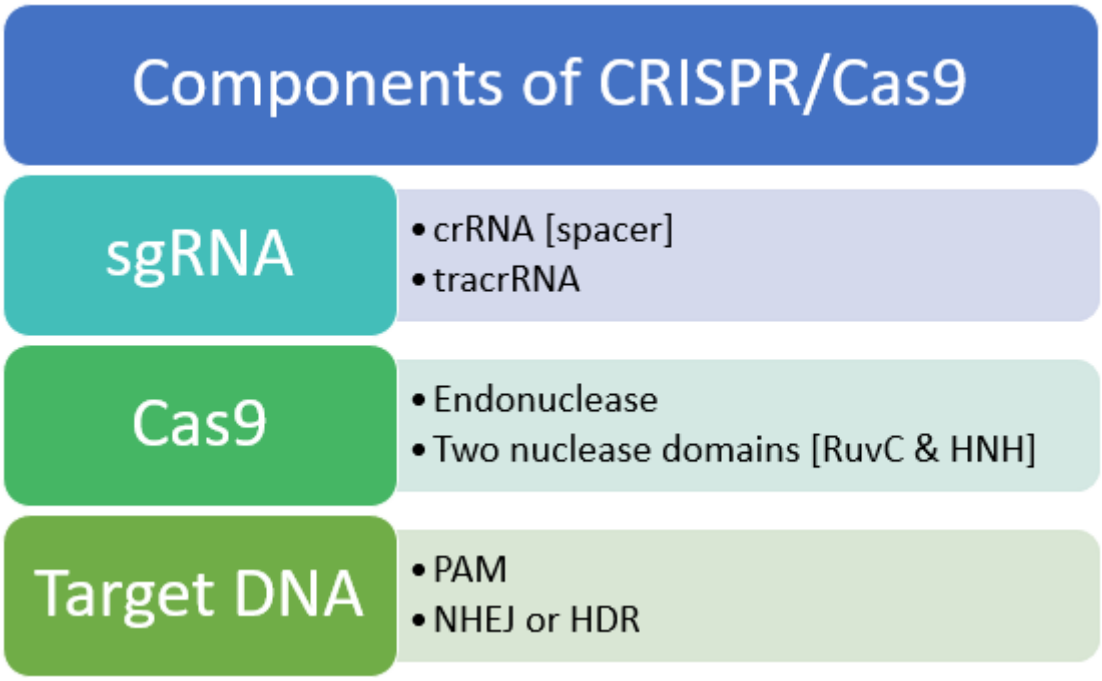

Figure 1. The main components of the CRISPR/Cas9 system. The gene-editing model requires single guide RNA (sgRNA) which functions as a probe to target its complementary foreign DNA sequence. sgRNA is a 20 nucleotide non-coding RNA sequence consisting of two parts: CRISPR RNAs (crRNAs) and trans-activating crRNA (tracrRNA). Cas9 is an endonuclease which has two nuclease domains (RuvC and HNH). Cas9 along with the sgRNA complex cuts the target DNA. Lastly the target DNA has the protospacer adjacent motif (PAM). Recognition of PAM by the sgRNA/Cas9 complex leads to unwinding of the target DNA, and the activation of Cas9's nuclease domains: HNH and RuvC domains. The double strand break in the DNA can be repaired through non homologous end-joining (NHEJ) by introducing insertions or deletions or homologous directed repair (HDR) used for recombining the genome

Overall, the objective of this application is to utilize it to modify undesired DNA sequences to repair or remove disease-causing alleles. Despite demonstrations of the successful studies of germline and somatic cell line genome editing via CRISPR/Cas9, there are issues that still must be addressed that raise ethical issues over the technique in clinical applications [4]. First, a method for safe delivery of the CRISPR/Cas9 vectors must be developed in order to successfully implement the system into the desired tissue(s). For years, geneticist have struggled utilizing these methods and delivery that have been complex and less successful than desired. Second, genome editing by CRISPR/Cas9 systems have demonstrated the creation of unwanted alterations of off-target sites. This may cause unwanted mutations in other sites even if the intended site is corrected, which may lead to more or bigger problems. Because of this, it is very important to improve CRISPR/Cas9 specificity.

The type II CRISPR/Cas9 system was modified in 2012 for the purpose to edit the genome in eukaryotic cells. Derived from *Streptococcus pyogenes*, the implementation of the prokaryotic immunity system into eukaryotic cells was accomplished by fusing crRNA with tracrRNA, forming a single guide RNA (sgRNA) [5]. This molecule, in turn, is used to recruit the Cas9 nuclease to the desired location within the genome. When injected in early stages of the embryo, the genome modification occurs in all cells, including germline cells, which are then passed onto future generations. Multiple studies have been conducted aiming at repairing a specific allele that cause a given disease. Wu et al. and his team conducted a study where they injected Cas9 mRNA and sgRNA into a mouse zygote whose target was the Crygc allele [6]. This allele is responsible for the formation of cataracts, a very common form of vision loss caused by clouding of the eye's lens. Implementing this technology into their study led to the successful correction of this mutation, resulting in a reduction of signs and symptoms in cataracts. As another example, a study demonstrated the successful correction of a mutation in the dystrophin gene, which is responsible for producing the protein that keeps muscle cells intact [7]. The mutation on this gene in particular is responsible for X-linked Duchenne muscular dystrophy (DMD), a type of muscular dystrophy. In this study, Cas9 mRNA, sgRNA, and single stranded oligo DNA were injected into a mouse zygote, but unlike the previous example, this attempt only achieved partial correction of the mutated gene. Speculation led to the authors of this study believing this was a consequence of injecting the Cas9 and other accompanying genes after the zygote stage and not before. Alternatively, this also may arise from two

different alleles responding differently to the CRISPR/Cas9 treatment.

Along with germline editing studies, somatic cell line manipulation has also been conducted with success. Type I tyrosinemia, an enzyme deficiency of fumarylacetoacetate hydrolase, is responsible for patients having the incapacity to metabolize tyrosine. In one of these studies, mouse models of disease mutation and phenotype for type I tyrosinemia were successfully corrected [8]. The CRISPR/Cas9 technology was implemented in this investigation. This was achieved by injecting Cas9, sgRNA and DNA oligo into the mouse liver. In succession, there was reduction in hepatocellular toxicity, and weight loss in the mouse model. Another study was conducted, *in vivo*, using an adenoviral CRISPR/Cas9 vector to cause mutations in the PCSK9 gene [9]. PCSK9 limits the uptake of low-density lipoprotein (LDL) cholesterol, and therefore alterations in PCSK9, accomplished by CRISPR/Cas9, led to successful increased uptake of LDLs. Due to the massive progression of this study, it is possible to begin using this application in clinical therapies in the near future.

CRISPR/Cas9 has also been applied in *ex vivo* studies, and one example involves the system to genetically alter β-thalassemia. Xie et al. utilized iPSCs (induced pluripotent stem cells) from fibroblasts of β-thalassemia patients and transfected them with CRISPR/Cas9 vectors which targeted the mutation that causes β-thalassemia [10]. The redesigned iPSCs were ultimately differentiated into normal red blood cells. This can potentially be used for an alternative treatment as opposed to transplantations. In another study conducted, via *ex vivo*, the CRISPR/Cas9 system was used as a two-part procedure involving its benefits in mitigating HIV. As HIV resides in memory T cells, the first step of treatment was to target HIV's long terminal repeats in the genome and eliminate it from the T cells using CRISPR/Cas9. This leads to the depletion of viral productions and reservoir. In the second step, genome editing was used to introduce a type of resistance gene into the non-viral genome to prevent HIV DNA from integration.

2. Delivery Methods Remain a Hurdle

Proper delivery methods for CRISPR technology to the target cell or organism have posed an obstacle to developing this technique for clinical application. To be therapeutically effective, the Cas9-gRNA complex must be directly delivered to the target cell's nucleus. While this has been achieved in the lab numerous times, research can only mimic clinical administration to a limited degree. Compared to treating a specific disease in an animal model or in an isolated cell culture with the application of CRISPR/Cas9, transforming the experience to *in vivo* is very difficult. By extension, most diseases require *in vivo* delivery of CRISPR/Cas9 for treatment and cannot be treated through *ex vivo* methods in the way that leukemia, for example, may be treated. As such, treating a disease at the genetic level differs significantly from managing its symptoms to treatment and diagnosis in its early stages of development.

In conjunction with Cas9's large size (160 kDa), the long phosphate backbone of gRNA, which bears a highly negative charge, results in a common obstacle to their application: crossing the cell membrane. Current delivery methods fall under two main categories: physical non-viral delivery and viral delivery. The former is safer, more cost-effective, and lacks the size limit characteristic of viral delivery; viral delivery, on the other hand, asserts higher delivery and infection efficiency in comparison.

Of the physical non-viral delivery methods, electroporation, nanoparticles, microinjection, hydroinjection, as well as mechanical cell deformation, are options that have been explored. Each method is defined by a unique set of advantages and disadvantages that shapes its development or lack thereof at the clinical level. This is would be most advantageous under the circumstances of cancer-causing genetic diseases, introducing new methods of therapeutic and medicinal delivery for cancer treatment **(Figure 2).**

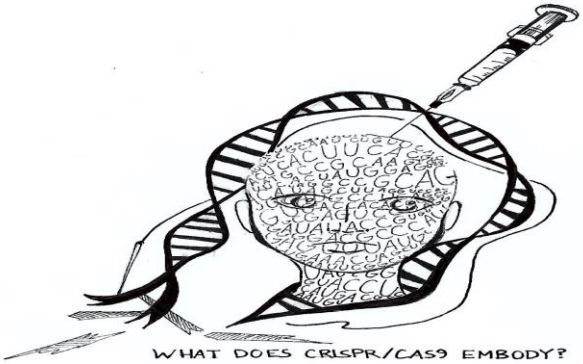

Figure 2. What does CRISPR/Cas9 embody?To some researchers, CRISPR/Cas9 represents a bold step forward in the direction of radical change; to others, a reason to step back and ponder its implications. Students of the sciences may view it as the embodiment of what it means to be specializing in a field replete with constant innovation, while the general population may continue on, unaware of its existence. The question, then, is—what does this technology represent and to whom?

2.*1. Electroporation*

Electroporation increases cell membrane permeability and is suitable for all cell types of the CRISPR/Cas9 system, being utilized widely in research on zebrafish regeneration, axolotl regeneration in embryonic cells, chicken development and mouse brain development [11]. Nevertheless, the desired genomic material is delivered to only 0.01% of target cells and the method induces significant cell death, yielding difficult in clinical implementation.

2.*2. Microinjection*

Microinjection, a direct delivery method usually into the pronucleus or nucleus of the cell, provides direct access to the target cells. Thus far, this method has been useful in evaluating overall gene-editing efficacy of different plasmid-based CRISPR/Cas9 systems targeting the same gene. However, it may cause unwanted side-effects such as cell damage, and requires a high level of sophistication and manual skills. Also, this is an inefficient technique given the large number of cells must be injected one at a time.

2.*3. Hydroinjection*

Another method, hydroinjection, is administered by rapid injection of nucleic acid solution into rodents, via their tail vein, in volumes of up to 8-10% of the rodent's body weight. This method has proved the most efficient in delivery of nucleic material to the liver and is widely utilized for the delivery of proteins, small interfering RNA (siRNA), DNA, and cancer cells. CRISPR/Cas9 complex has also been successfully delivered using this technique; as a result, it holds promise in establishing and developing liver cancer models for further research and may pave a smoother path to transitioning CRISPR/Cas9 into the clinical setting, albeit under limited applications. Its success thus far has been generally limited to small animals, whereas in large animals, data shows increases in blood pressure, temporary cardiac dysfunction, liver expansion, and in some cases, organism death -- a negative outcome that bodes ill for any current or near future application to treat humans.

2.*4. Mechanical Cell Deformation*

Other delivery techniques have shown promise *in vitro* but have yet to be adapted *in vivo*, such as mechanical cell deformation. In lymphoma cells, this method demonstrated a 70% knockout efficiency. Since lymphoma cells are especially difficult to be transfected, this method holds a high potential in treating this type of cancer.

While the relative safety of physical delivery methods encourages their use and development for CRISPR/Cas9 application, their poor delivery efficacy limits their use *in vivo* and stunts their establishment in the clinical setting. As a result, their application is typically limited to the research setting, in generating knockout cell lineages and animal models of various diseases and conditions. Viral delivery, on the other hand, has been the choice of gene-editing delivery for approximately thirty years and its accuracy, as well as safety, has been the subject of refinement for many of those years. Regardless of its off-target effects and initiation of immune response-- disadvantages and safety concerns of this method-- viral vectors boast higher rates of targeting and integration, which remain to this day the prime method of delivering genomic material, as well as the CRISPR/Cas9 complex, to cells. Currently, two main viral delivery methods are in use with regards to CRISPR/Cas9 technology: adeno-associated viral vectors, and lentiviral vectors.

1.5. Adeno-Associated Viral Vectors

Adeno-associated viral vectors offer a suitable method for delivering the CRISPR/Cas9 complex to cells due to their broad range of serotype specificity, their ability to infect dividing and non-dividing cells, as well as their combination of non-pathogenicity and mild immunogenicity. In fact, Europe approved the use of Glybera, an AAV-based gene therapy, to treat patients with lipoprotein lipase deficiency, indicating its efficacy and safety in clinical application to humans. While this milestone highlighted the promise that AAV vectors hold as carriers for gene therapy technologies, AAV vectors still require refinement before applied medically, due to their packaging limit of approximately 4.5 kb. A possible alternative that has been posed to this obstacle is the use of truncated SpCas9 or *Staphylococcus aureus* (SaCas9), which demonstrates similar gene editing efficiency but lower activity. Furthermore, a dual AAV delivery system, which separately delivers Cas9-encoding DNA and sgRNA to overcome the packaging limit of using only one AAV, is another possible solution. This method may be feasible for the therapy of metabolic liver disease in animal models.

1.6. Lentiviral Vectors

Mild immunogenicity, long-term expression of transduced genes and high infection efficiency in non-dividing cell are all advantages that recommend lentiviral vectors for use as delivery vectors of the CRISPR/Cas9 complex, as well as other gene-therapy methods. Moreover, lentiviruses have also shown successful delivery results *in vivo* and *in vitro* for gene therapy. In combination with CRISPR/Cas9, these vectors demonstrate potential in performing function-based screening in mammalian cells, generating knockout animal models, establishing animal models of cancer such as acute myeloid leukemia (AM), and eradicating potential viral infections such as latent Epstein Barr virus genomes in Burkitt's lymphoma cells.

Due to its large size as well as the specificity of the cell membrane, *in situ* production of Cas9 within the cell has emerged as a viable and alternative option in administering the CRISPR/Cas9 complex to the cell. This method can be achieved by encoding Cas9 in either DNA or mRNA form through plasmid-based delivery, which is both easy and inexpensive. However, while

this method evades the cell membrane rejection, other factors must be considered such as the nature of the promoter or replication sequence for Cas9 which is unsuitable within mammalian cells if it is of bacterial origin. Using plasmids also increases expression time, which may be beneficial for sustained expression of CRISPR/Cas9 if that is an intended part of the therapeutic regimen, but this may increase the occurrence of off-target effects. Furthermore, plasmid delivery has been associated an immunogenic response. Encoding the Cas9 protein in mRNA instead of DNA is a possible solution to attenuate the immunogenic response. Studies show that Cas9 mRNA delivery resulted in swifter gene-editing due to immediate translation of the genetic material, whereas with DNA delivery the nucleic material must first be transcribed into mRNA and then translated, yielding a slower process and more room for off-target effects. Nevertheless, mRNA delivery is less stable than DNA and is prone to degradation by RNases *in vivo* and *in vitro*.

Chemical delivery of the CRISPR/Cas9 complex, whereby the deliverable is modified to make it more likely to cross the cell membrane and avoid degradation by the target cell, offers a possible solution to these obstacles. Lipid encapsulation and the use of gold nanoparticles are examples of this technique and may be refined for use. Also, receptor-mediated delivery using *S. pyogenes* Cas9 proteins harboring asialoglycoprotein receptor ligands (ASGPrL) is another method that has shown success in recent research, providing a possible approach for cell-selective gene-editing in the future.

3. It's Progress in Therapeutic Techniques:

CRISPR/Cas9 has enabled an era where scientists hold the key to curing many genetic abnormalities. While introducing this genetic tool to engineer human cells in the desired ways is ultimately the goal, research has proven time and time that the CRISPR/Cas9 technology is not different from any other scientific techniques and, as a result, the list of diseases scientists seeks to remediate through the application of CRISPR/Cas9 remain similar. Viral diseases such as HIV-AIDS, HPVs, and HBV as well as inherited genetic disorders such as cystic fibrosis and beta-thalassemia represent candidates in CRISPR's target inventory.

3.1. Inherited genetic disorders

Inherited genetic disorders represent primary candidates in CRISPR/Cas9's target inventory. Correction of causative mutations by genome editing is especially effective for monogenic disorder such as cystic fibrosis and β-thalassemia. Providing a broad view of the therapeutic applications of CRISPR/Cas9 and its great potential, Wojtal et al. applied this innovative system to a variety of inherited genetic disorders, garnering surprising success and setting a foundation that subsequent researchers can build on [12]. In their study, they successfully upregulated utrophin production in skeletal muscle cells of a patient with Duchene Muscular Dystrophy, providing an efficient approach at compensating for the loss of dystrophin with progression of the disease [12]. Furthermore, they demonstrated that a specifically designed PAM-discriminating single-guide RNA preferentially targets the mutant allele characteristic of achondroplasia, the main cause of dwarfism [12]. Their application of CRISPR/Cas9 also extended to *ex vivo* removal of a large genome rearrangement within a male individual diagnosed with MECP2 duplication syndrome, an x-linked disease that causes macrocephaly and intellectual disability [12]. Unwanted side effects were not observed, although the off-target mutations were not checked thoroughly. Their research covers a broad range of potential applications related to CRISPR/Cas9 and demonstrates the immense possibilities associated with a gene-editing technology with the capacity to be adapted to a variety of inherited genetic disorders, some of which will be discussed in this section.

3.2. Mitochondrial Disease

CRISPR/Cas9 has also emerged as a possible curative candidate for mitochondrial diseases. With little treatments to speak of, mitochondrial diseases affect families in a devastating manner, often causing infant death immediately upon birth [13]. As of yet, transmission prevention is the only avenue by which the inheritance of mitochondrial diseases is deterred. While mitochondrial replacement therapy has proven effective in combatting the daunting effects caused by mitochondrial disease, especially for mothers of advanced maternal age who carry lesser quantities of mutant mtDNA in their eggs, it carries the stigma of being termed a "three-parent" treatment [13]. In contrast, CRISPR/Cas9 treats the embryo at the blastocyst stage, before implantation into the uterus, at the integral time during which it is being diagnosed, furthermore, CRISPR/Cas9 is not burdened by the ethical controversy of being the "three-parent" treatment. On the other hand, CRISPR/Cas9's potential to be directly and efficiently applied to alter the human germline has been met with ample doubt, scrutiny and calls for caution. Stressing the urgent need for more research, interdisciplinary discussion, and a clear, unbiased understanding of its function and aptitude.

Also, worth noting is the nature of a progressive study conducted by Liang et al. in 2015 where the authors employed CRISPR/Cas9 gene-editing technology to edit the HBB-gene which codes for the human beta-globin, in human tripronuclear zygotes [14]. While their results represented a certain, albeit limited, degree of confidence and efficacy for developing this novel technology for use in human medical treatment, it also highlighted the lack of knowledge concerning off-target effects and, by extension, the need for refinement and improvement in the field especially when considering its application to humans [15].

3.3. Cystic Fibrosis

Cystic fibrosis, a life-threatening genetic disease, causes thick mucus to form in the lungs, impairing breathing and providing nurturing grounds for a variety of pathogens [16]. Current treatments for the lung disease, a leading cause of death around the world, focus on preventative and management measures [16]. However, CRISPR/Cas9 offers an avenue by which permanent treatment may be offered to patients. As such, Firth et al. demonstrated the successful use of CRISPR/Cas9 in targeting a mutation in the CFTR gene—the homozygous deletion of F508—in iPSCs. The corrected iPSCs were subsequently differentiated into mature, functional airway epithelial cells where correct CFTR expression was observed [16].

In another study, Schwank et al. corrected the expression of the CFTR gene locus in intestinal epithelial cells in adult patients with cystic fibrosis using homologous recombination in cultured intestinal stem cells [17]. Their work provides insight into methods with promise for single-gene defects and sheds light on the possibilities associated with treating human patients through use of this technology [18].

Furthermore, on the research front, Dow proposes using pigs to model the progression of monogenic disorders such as cystic fibrosis [19]. While not necessarily the primary choice in modeling such diseases, due to cost, size and obstacles to introducing targeted mutations, their physiology resembles that of humans and using CRISPR/Cas9-induced mutations, pigs could emerge as valuable models in preclinical studies [19].

3.4. Sickle Cell Anemia

A single nucleotide substitution—a thymine for an adenine—in the beta globin gene, results in the sickle cell disease, causing immense suffering, and often, premature mortality. Currently, hematopoietic stem cell transplantation as well as chronic therapies, such as hydroxyurea [21], is used to treat patients with this disease. However, the need for a curative therapy that can be adapted to all patients without worry about the under-representation of minorities in the stem cell donor registry and one that can focus on corrective rather than preventative measures emerges [20]. As a result, the focus has shifted to improving gene-editing techniques, primary among them CRISPR/Cas9. In this regard, Hoban et al. successfully applied CRISPR/Cas9 to CD34+ cells resulting in over 18% modification of the mutation *in vitro*, and a subsequent restoration of wild-type hemoglobin in the CD34+ stem and progenitor cells obtained from the bone marrow of sickle cell patients [22].

Furthermore, DeWitt et al. utilized a combination of a single-stranded DNA oligonucleotide donor with an unmodified sgRNA to correct the sickle cell mutation in human hematopoietic stem and progenitor cells (HSPCs), resulting in reestablishment of wild-type hemoglobin and an overall reduction of sickle cell RNA and protein observed in differentiated erythroblasts [23]. When Dewitt et al. introduced CRISPR/Cas9-corrected HSPCs into immunocompromised mice, maintenance of the gene corrections well into 16 weeks was successfully observed [23]. Their results represent a possible avenue by which CRISPR/Cas9 may be applied to the clinical setting in treating compromising disorders such as sickle cell disease [23].

Contrastingly, using an alternative approach, Ye et al. induced targeted deletion of 13 kilobases within the beta-globin gene to imitate the Sicilian mutation characteristic of hereditary persistence of fetal hemoglobin HPFH—a condition in which individuals exhibit high quantities of fetal hemoglobin throughout life [24]. They observed 31 % targeted deletion in cells after gRNA delivery and noted increased γ-globin in HSPCs with deletion compared to those without [24]. Coupled with sickle cell anemia or, by extension, β-thalassemia, this genomic modification lessens the painful symptoms characteristic of individuals that are homozygous for these diseases by inhibiting sickling [24, 25]. Interestingly, in an evaluation of TALENs and CRISPR/Cas9 nuclease system to correct the sickle cell mutation, Hoban et al. discovered that while both techniques led to successful gene modification, CRISPR/Cas9 led with 37% modification in K562 cells compared to TALENs' 18% [26]. ZFN was not included in this comparison, but given the similar or even classic nature of ZFN compared to TALEN [1], CRISPR/Cas9 will be the first choice in future applications.

3.5. Beta-Thalassemia

As a common genetic blood disease with over 300 genomic causes resulting from absent or reduced beta globin chains in hemoglobin, β-thalassemia is included in the inventory of diseases targeted by CRISPR/Cas9 [27, 28]. Currently, gene therapy, patient-specific iPSCs, and hematopoietic stem cell (HSC) transplantation methods are used to treat this complex disease [27]. Moreover, with the development of highly intricate gene-editing technologies, management of β-thalassemia in patients will greatly depend on the stratification of subgroups defined by the myriad of causes and influences, whether disease modifiers or primary mutations [28]. Nevertheless, harmful side effects, obstacles to homologous recombination, as well as a limited number of HLA-matched donors represent drawbacks to these approaches [27]. As a result, CRISPR/Cas9 represents a promising curative technique for treating this disorder. In a surprising study, Yang et al. reprogrammed fibroblasts of a patient with beta-thalassemia into naïve iPSCs, which are characterized by much higher rates of self-renewal ability [30]. Furthermore, when used with CRISPR/Cas9, they demonstrated superior aptitude for gene-correction in comparison to conventional primed iPSCs [30].

On the other hand, Xie and colleagues, using CRISPR/Cas9 technology combined with the *piggyBac* transposon, successfully corrected the HBB mutation observed in patient-derived iPSCs [29]. Their results

indicated that no off-target effects were observed and full pluripotency, as well as a normal karyotype defined the resultant cells [29].Utilizing single-strand oligodeoxynucleotides (ssODNs) in combination with CRISPR/Cas9 nucleases Liu et al. successfully corrected the β-41/42 (TCTT) deletion mutation in iPSCs of patients with Beta-thalassemia [31]. When analyzed, no off-target mutagenesis was observed and, furthermore, the corrected cells exhibited normal beta-globin transcripts when differentiated into erythroblasts [31].

Recently, in a revealing comparison between TALENs and CRISPR/Cas9, Xu et al. employed both gene-editing technologies to target the intron2 mutation site IVS2-654 loci in the globin gene [27]. Their results indicate a higher homologous targeting using TALENs in comparison to CRISPR/Cas9 [27]. Furthermore, they observed higher potential for off-target effects in CRISPR/Cas9 than in TALENs demonstrating that six sites showed indel formation where CRISPR was targeted in comparison to TALENs' two sites [27].

3.6. Huntington's Disease

Characterized by dementia, psychiatric, and behavioral instability, Huntington's disease, although a rare neurodegenerative disorder, causes patient suffering and pains those witness to its onset and progression. With a focus on inactivating only the deleterious form of the allele, Shin et al. successfully excised approximately 44 kilobytes of the DNA spanning the CAG repeat expansion mutation, among other areas, such as the promoter region of the mutant HTT gene using CRISPR/Cas9 [32]. Further testing and analysis revealed complete prevention of mutant mRNA and protein production [32]. Their success demonstrates the power and specificity of using sequences unique to Huntington's disease haplotype in relation to CRISPR/Cas9 technology. Furthermore, in a monumental proof-of-study, Dr. Merienne and his colleagues reduced the aggregation of Huntington's in the mouse striatum of the brain by altering the reading frame of the HTT gene through the application of CRISPR/Cas9 [33, 34]. While, at the time that the experiment was conducted, he and his colleagues hadn't analyzed any behavioral changes. The success demonstrated by his research provides a template by which other neurodegenerative and hereditary movement disorders may be treated [33].

In another study, Xu et al. combined CRISPR/Cas9 technology with the *piggyBac* transposon producing an approach that corrected isogenic induced human pluripotent stem cells with Huntington's disease for phenotypic abnormalities such as impaired neural rosette formation as well as deficits in mitochondrial respiration [35]. They also showed that any differences between HD and normal healthy controls were absent when tested for between HD and the corrected iSPCs cell lines [35]. Additionally, Monteys et al. designed guide RNAs in an experiment that provided illuminating insight into the nature of allele-specific binding. They identified prevalent PAM sequences which either interfere with or create a PAM sequence necessary for CRISPR/Cas9 targeting activity [36]. Their results reveal that the distance between the upstream and downstream guide influenced corrective efficiency and that indels within the first intron of the sequence lessened overall expression of HTT [36]. As a result, consideration for the effects such indels may have on the normal allele is paramount during the design of guide sequences.

3.7. Hemophilia

Hemophilia, a monogenic x-linked disorder in which the blood lacks the necessary coagulator factors, results in severe bleeding episodes and bruising. Without any permanent cure to speak of, this disease disables the patient greatly. For this reason, researchers are currently developing and improving several gene-editing techniques to treat this illness, chief among them—CRISPR/Cas9.

In an elegant experiment, Guan et al. generated a mouse strain with the F9 missense mutation identified in a male patient diagnosed with Hemophilia [37, 38]. Subsequently, they introduced *in vivo* correction through hydrodynamic tail injection of cas9 protein and the respective sgRNA with the desired ssODNs or long donor DNA. As a result, they observed 0.56 % correction with the use of ssODNs and 1.5% correction with the application of long donor DNA in the hepatocytes, indicating greater success with the use of long donor DNA which could guide future use and application of CRISPR/Cas9 technology [37, 38]. A number of indel formations were observed in which the corrected DNA was not inserted, although hepatocyte damage was not reported [38]. Furthermore, in the employment of two adenoviral vectors to deliver the corrected form of the gene, Guan et al. reported higher rates of hepatocyte correction at 5.5% during the early stages of observation, followed by virtually no correction of clotting at later time points [37, 38]. This discrepancy is due to inflammation of the liver and significant hepatocyte death, which demonstrates a need for considering the immunogenic effects of viral vectors when delivered with CRISPR/Cas9 systems [38].

On the other hand, Park et al. demonstrated correction of Hemophilia A in a potentially lethal mouse model by correcting the characteristic chromosomal inversions at sites intron 1 and 22 in the F8 gene of derived iPSCs using CRISPR/Cas9 nucleases [39, 40]. Resultant iPSCs were isolated with correction frequencies up to 6.7% and with no display of off-target mutagenesis. Subsequent differentiation of endothelial cells from the corrected iPSCs demonstrated successful F8 gene function [39, 40].

Further, providing advanced avenues and resources by which research can be enhanced, Qihan et al. obtained F *IX* knockout mice strains with Hemophilia B using the CRISPR/Cas9 system [41]. From a population of 60 mice,

they identified an 85% mutation rate and subsequently observed no off-target mutations [41]. Their work expands the body of resources available to researchers by using cutting edge technology to produce readily available study models cheaply and efficiently for furthering the study of disease.

3.8. Duchene Muscular Dystrophy

Lacking an effective treatment, the X-linked disease, Duchene Muscular Dystrophy is caused by a mutation in the gene encoding the dystrophin protein, which is vital to muscle fiber strength and integrity. With the progression of the disease, the patient skeletal muscles weaken considerably and the ability to walk may be lost. Currently, CRISPR/Cas9 research is heading the front to treat this disease by analyzing and altering its expression at the molecular and genomic level. In 2014, an early experiment conducted by Long et al. applied CRISPR/Cas9 to edit the germline of *mdx* mice models with Duchene Muscular Dystrophy (DMD), producing genetically mosaic mice displaying restored dystrophin function within the range of 2% to 100% [42]. They also observed that complete phenotypic rescue of muscle integrity in mice depended on only a subset of corrected cells *in vivo*, highlighting the influence of corrected cells in muscle renewal [42]. On the other hand, a number of researchers have utilized CRISPR/Cas9 in tandem with adeno-associated viral vectors to excise the mutated form of exon 23 in the dystrophin gene within the *mdx* mouse models [43, 44]. Upon testing, this deletion was found to successfully induce expression of dystrophin and also to partially restore dystrophin function in cardiac and skeletal muscle fibers [43, 44].

For example, Bengtsson et al. delivered CRISPR/Cas9 components through AAV6 application to correct dystrophin expression in mdx^{4cv} mice models [45]. They restricted Cas9 expression to cardiac and skeletal muscle using the CK8 regulatory cassette, thus demonstrating an improvement in methods that avoid inducing off-target mutagenesis, and displaying induction of dystrophin expression, as well [45]. Moreover, in a study targeted at 60% of those diagnosed with DMD, Young and colleagues successfully employed CRISPR/Cas9 to delete 725 kilobytes of DNA data in hiPSCs, thus reframing the DMD gene and restoring the dystrophin protein in analyzed cardiomyocytes and skeletal myotubes [46]. Furthermore, they observed that, despite the deletion, the dystrophin remained functional and membrane integrity had been enhanced [46]. Their results indicate the valuable possibility of looking at cures for diseases in terms of reframing their sequence rather than solely deleting the deleterious form to introduce the wild-type functional sequence.

In an alternative study, Long and colleagues injected *mdx* mice with adeno-associated virus-9 (AAV9) *in vivo* with gene-correction sequences at particular postnatal intervals, namely postnatal day 1, day 12 and day 18 [47]. At each interval, the researchers applied a different mode of delivery including intraperotineal injection (P1), intramuscular (P12), and retro-orbital (P18), respectively [47]. Their data indicated that most of the modes yielded positive dystrophin expression in cardiac and skeletal muscle, with a steady increase throughout the following 12 weeks demonstrating continued expression of the protein [47]. In a recent experimental approach, Lattanzi et al. applied CRISPR/Cas9 to myogenic cells from eight patients with the common exon 2 duplication to induce deletion of this mutation using only one guide RNA (gRNA) with the intention of reducing off-target effects [48]. Dystrophin expression was successfully restored, with approximately 73% on-target activity in 110 tested clones [48]. Moreover, off-target effects were not detected by T7E1 assay [48].

3.9. Inherited Retinal Diseases

The death or dysfunction of photoreceptors characterizes retinal degenerative diseases and leads to severe and, often, incurable vision loss [49]. However, CRISPR/Cas9 heads the gene-editing endeavor to provide a solution to this unfortunate situation [49]. In a proof-of-principle experiment, Bakondi and his colleagues demonstrate improvement of visual function in rat models of severe retinal degeneration employing CRISPR/Cas9 to selectively ablate the rhodopsin gene carrying the dominant S334ter mutation *in vivo* [50]. *Furthermore, employing a personalized iPSCs-based approach, the RPGR mutation causing X-linked retinitis pigmentosa was corrected in 13% of gene copies by Bassuk and colleagues [51]. They transduced donor induced pluripotent stem cells from a patient diagnosed with photoreceptor degeneration using rna-guided CRISPR/Cas9 and reported percentage correction levels comparable to experiments of a similar nature [51]*. In another study, Ruan et al. designed a self-limiting CRISPR/Cas9 system to reduce exposure to SpCas9 expression which could be harmful [52]. Using this system, the intronic mutation in CEP290 characteristic of LCA10, a subtype of Leber congenital amaurosis, was successfully targeted and removed, thereby introducing wild-type expression of CEP290 [52]. Moreover, in an in-vivo experiment, Latella et al. delivered a plasmid carrying the cas9 protein and desired sgRNAs through subretinal electroporation to edit the human rhodopsin gene, responsible for normal vision, in a P23H transgenic mouse model of autosomal dominant Retinitis Pigmentosa [53]. While their results demonstrated success in reducing the frequency of the P23H dominant mutation, they identified a lower rate of knockout *in vivo* compared with their previous *in vitro* results [53]. Possible causes for this discrepancy as identified by the researchers are a lower number of Cas-9 bearing plasmids delivered *in vivo* than *in vitro* and incomparable differences in knockout and editing efficiency intrinsic to both processes [53].

Using CRISPR for another approach, namely identifying the causative gene variant responsible for

retinal degeneration in rodless mice models of retinitis pigmentosa, was a task undertaken by Wu et al [54]. Their research effectively resolved the debate surrounding the exact cause of degeneration in the mice which are homozygous for the following two mutations: the nonsense Y347X mutation and the Xmv-28 intronic insertion of the leukemia virus. Analyzing first and second generation CRISPR-repaired mice revealed the nonsense Y347X mutation as the cause underlying initiation and progression of the disease [54]. Their results provide integral insight into treatment for retinal degeneration, especially since the mutated Pde6b gene in mice has a comparable intron to exon relationship with its PDE6B ortholog in humans [54].

3.10. Viral diseases

CRISPR/Cas9 is redefining how the medical and research-based settings are viewing the treatment of disease. By targeting the key genetic elements responsible for the initiation and proliferation of viral disease, CRISPR/Cas9 has broadened the understanding of the fundamental features informing the progression of several viral diseases such as HIV, hepatitis B and even human papillomaviruses responsible for cancer. However, CRISPR/Cas9 screens have also illuminated the cellular factors responsible for facilitating or restricting viral genome replication [55]. In tandem with its use as screens, libraries could possibly be generated carrying clones of human cell lines each lacking a gene vital to viral replication [55]. In this way, research between different labs may be unified through the application of one genetic library [55].

3.11. HIV-AIDS

Acquired immune deficiency syndrome (AIDS) is caused by human immunodeficiency virus (HIV) infection. However, it is impossible to eliminate HIV from the human genome, making it impossible to cure HIV. While current drug applications suppress HIV-1 replication and subsequent activity, they are not capable of eliminating entirely the responsible provirus. Nevertheless, CRISPR/Cas9 gene-editing methods represent a new era of capability regarding the treatment of incurable viral disease. In a recent study, Limsirichai et al. demonstrated that CRISPR-based transcription activators can be employed to activate gene expression within latent HIV-1 reservoirs [56]. In combination with antiretroviral therapy, this strategy presents a powerful form of treating transcriptionally inactive proviruses [56]—a notable alternative to expensive TALENs and ZFNs systems [57]. Another study conducted by Wang et al. revealed that HIV-1 was consistently able to escape replication inhibition through a series of escape variants including indel formations and substitutions detected around the targeted cleavage site [58]. Further study revealed that the introduction of these mutations is a typical hallmark of the nonhomologous end-joining (NHEJ) pathway characteristic of the mammalian cell [58].The subsequent dynamic highlights a twofold pathway CRISPR/Cas9-mediated treatment may follow—inactivation of the virus or acceleration of viral escape through the induction of mutations that prevent CRISPR/sgRNA recognition or that reintroduce replication competent viruses into the host [59]. To circumvent this obstacle, Wang et al. employed a dual gRNA combination that blocks viral escape [60]. As a result, repeated Cas9 cleavage in the target site saturated critical mutations that blocked protein production and replication competence [60]. Moreover, in a remarkable study, Kang et al. identified the chemokine receptor 5 (CCR5) as a key component worthy of consideration in the endeavor to cure HIV-1 [61]

. Serving as an HIV-1 coreceptor, recent research has shown that lack of CCR5 results in protection against contraction and infection by HIV-1 [61]. Furthermore, a leukemic, HIV-positive patient treated with hematopoietic stem cells containing a CCR5 mutation resulted in a cure for both HIV and leukemia [61]. With attention to the safety and efficacy of CRISPR/Cas9 delivery systems [57], elucidating the role CCR5 mutation plays in protecting against HIV-1 infection potentially allows for a cure that could circumvent initial contraction of the disease, thereby focusing on preventative measures as well as treatment.

3.12. Cancer

The CRISPR/Cas9 system has also enabled the study of cancer on an increasingly minute level. While many studies indicate success in the development and observation of carefully selected cell lines, CRISPR/Cas9 has enabled insight into the TERT locus, whose expression aids in the determination of human telomerase activity [62]. Which, in turn, is responsible for adding nucleotides to telomeres, especially in cancer cells. Occurring in extremely small numbers per cell, current methods for identifying and investigating TERT function are inefficient [62]. CRISPR/Cas9 genome editing, on the other hand, mediates the tagging of the TERT protein with an easily identifiable epitope tag, which enables further study of telomere structure [62]. As known cancer-causing agents, high-risk human papillomaviruses HPVs 16 and 18 HPV-16 and HPV-18 are responsible for many infection-caused cancers in female patients worldwide, compared to a considerably lower 5% rate in males [63]. Moreover, they are also responsible for several head and neck cancers [63].

As a result, current and ongoing research utilizing the CRISPR/Cas9 system targets HPV-16 and HPV-18 in tandem with the associated HPV oncogenes E6 and E7, which are responsible for degrading p53, a tumor suppressor, and for destabilizing the Rb protein, respectively [64]. In an earlier experiment applying CRISPR/Cas9 technology for the inhibition of cell lines infected with HPV, Kennedy et al. induced indel mutations in the E6 and E7 oncogenes resulting in their inactivation [64]. Their data indicated subsequent expression of p53 and Rb, resulting in cell death [64]. This proof-of-principle experiment provided the pivotal

grounds for similar research to come later. In a similar experiment, Zhen et al. transduced CRIPSR/Cas9 targeting promoter HPV-16 E6 and E7 cervical cell lines, thereby reducing proliferation of cancer-positive cells through the accumulation of p53 and p21 proteins *in vitro* [65]. Furthermore, subcutaneously inoculating these cells into nude mice, the researchers identified inhibited tumorigenesis [65]. Moreover, in an experiment utilizing the CRISPR/Ca9 system, Yu et al. demonstrated cleavage of HPV16-E6, leading to cell apoptosis and subsequent inhibition of growth in SiHa and CaSki cells bearing this virus [66]. They also observed reduction of the E6 protein coupled with renewed p53 expression [66].

On the other hand, Hu et al. identified similar results in their research targeting HPV16-E7 in positive cell lines [67]. By disrupting expression of this oncovirus, Hu et al. demonstrated the efficacy of applying CRISPR/Cas9 technology to inhibit growth and induce apoptosis within cells positive for HPV expression [67]. In a study aimed at improving the efficacy and safety of delivery of the CRISPR/Cas9 vehicle and its corrective contents, Ehrke-Schulz and Ehrhardt engineered gene-deleted high capacity adenoviral vectors with HPV-specific sgRNAs and Cas9 [68]. Their data demonstrated powerful targeting of HPV-positive cervical cell lines resulting in cell death, increased apoptosis in comparison to control groups, as well as decreased viability of these cells overall [68]. They posit that, high-capacity adenoviral vectors (HCAdVs) can be utilized in the lab and medical settings as powerful oncolytic agents [68]. In an interesting study, Zhen et al. combined CRISPR/Cas9 targeting the E6 and E7 oncogenes characteristic of HPV-16 with cisplatin cis-diaminedichloroplatinum II or CDDP, establishing a powerful dynamic whereby CRISPR/Cas9 acted as a chemosensitizer, inducing a pro-apoptosis effect in the cervical cancer cells [69]. Their results also revealed that the combination of both methods was superior than using either on its own, thus paving the way for viewing CRISPR/Cas9 not just as technology to be applied solely on its own, but also as a supplement that can improve current methods [69].

3.14. Hepatitis B Virus (HBV)

An estimated 240 million people worldwide are infected with Hepatitis B virus, or HBV, which functions by reverse transcription [70]. Current treatments for the disease include oral nucleotide and nucleoside analogs which target the viral reverse transcriptase to inhibit the synthesis of DNA [70]. However, mere weeks to months after withdrawal of treatment, the virus may reactivate, once more putting HBV patients on lifelong NUC therapy, which, unfortunately, nurtures the rise of resistance through mutation selection [70]. Considering the current state of treatment, researchers are honing CRISPR/Cas9 use for application to HBV. As such, in one of the most successful experiments to date, Ramanan and his colleagues reduced cccDNA by an approximate 92% in cell culture [71]. In fact, cccDNA reduction continued to progress from an estimated 71% by Day 21 to an estimated 92% reduction, 15 days after initial transduction [71]. Interestingly, their findings suggest that no significant reduction in the levels of HBV DNA was rendered [71]. Furthermore, in a proof-of-principle experiment, Kennedy et al. successfully demonstrated downregulation of HBV cccDNA using bacterial Cas9 and HBV-specific sgRNAs through the use of lentiviral transduction [72]. Their results indicate that the HBV DNA production was inhibited within *in vitro* models and that overall viral genome levels were reduced by approximately 1000-fold compared to original levels [72]. Moreover, they also successfully reduced cccDNA levels by about 10-fold providing foundational evidence and data for steering this technology towards treatment for those chronically infected with HBV [72].

In a recent experiment, Li et al. excised a full-length integrated HBV DNA fragment composed of 3,175 base pairs, leading to successful destabilization of cccDNA in an otherwise stable HBV cell line [73]. Their novel findings represent a turn in the direction towards a radical cure presenting complete eradication of the HBV infection from a stable cell line [73]. Combining an all-in-one CRISPR/Cas9 system with co-transfection of a plasmid expressing HBV resulted in the substantial reduction of surface and envelope antigens expressed by the virus by Sakuma et al [74]. In addition, they confirmed successful fragmentation of the HBV genome while also noting that a rare number of observed off-target mutations were identified only with deep-sequencing analysis [74]. Nevertheless, and acknowledging the many successes made in treating HBV with CRISPR/Cas9, many researchers continue to stress that, being proof-of-concept experiments, CRISPR technology still requires robust improvement regarding a number of issues such as off-target effects and whether it can be honed to target all infected cells within the host [70]. Given that HBV DNA can be identified in various tissues and viral reservoirs throughout the body, it is paramount that for complete treatment and, furthermore, full eradication of the disease, the endonucleases utilized in CRISPR/Cas9 therapy are targeted at every infected cell [70] **(Figure 3).**

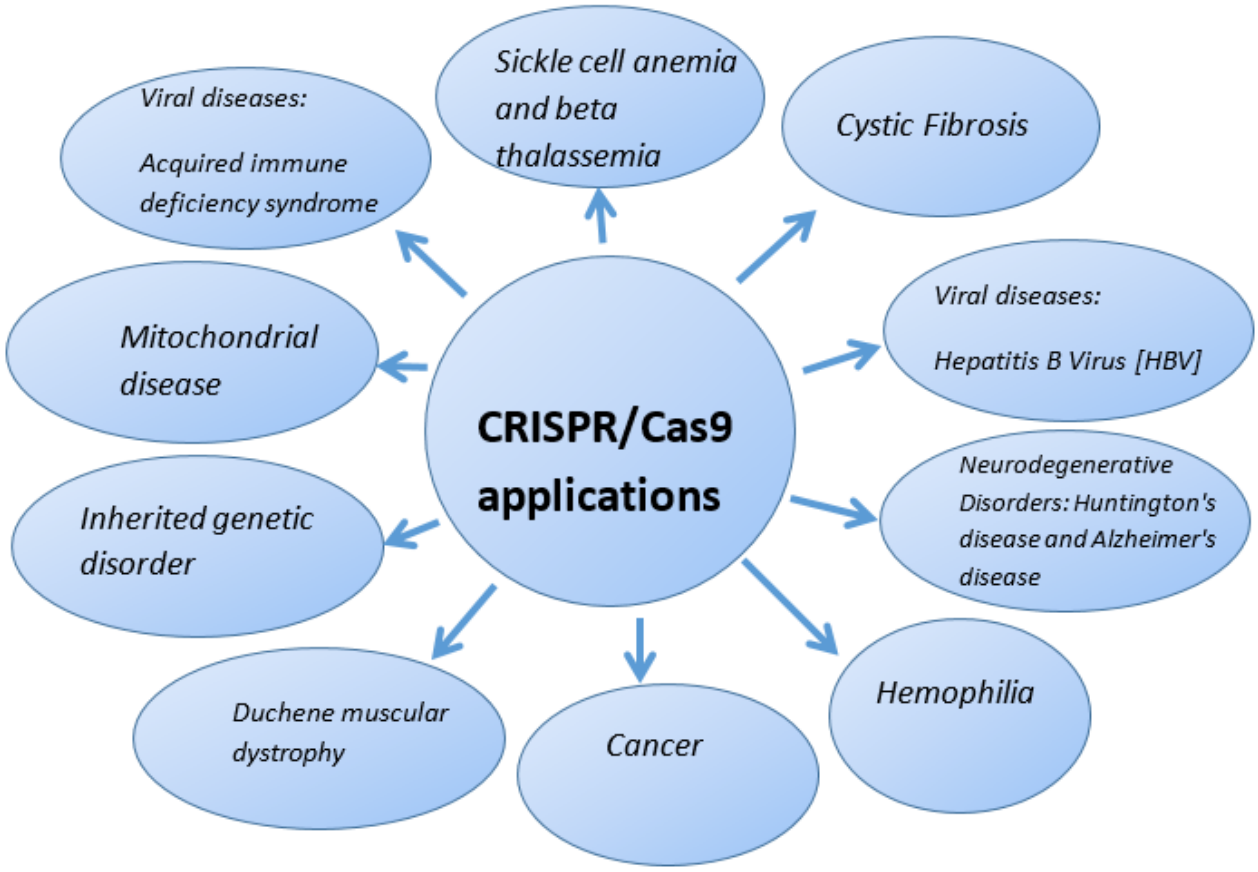

Figure 3. Practical Uses of CRISPR/Cas9

4. A Budding Technology in Need of Refinement

CRISPR technology's ability to efficiently and inexpensively target genomic aberrations for correction defines it as a key player in the development of personalized medicine in the future. There have already been advancements in delivery methods involving this form of technology ranging from electroporation to lentiviral vectors. Because of the unique nature of targeted genes there are various methods utilized in delivery of this technology. Some methods are proven to be more effective than others under certain conditions that are most ideal and less expensive. There has already been some research introducing CRISPR into several forms of ailments that involve genetic faults. Those including sickle cell anemia, hemophilia, as well as research involving various cancers. The implementation of CRISPR technology can produce adverse effects in malignant diseases, reducing the rates of fatality and morbidity.

Despite this promise, many issues must be addressed, namely its off-target effects, the efficacy of CRISPR delivery methods and vectors, as well as its interaction with p53 tumor suppressor within the cell. The application to preventive medicine is another issue to be discussed. Editing human genome for the prevention purpose should be ethically approved when the locus causes severe genetic disease. However, this attempt easily leads to the 'human enhancement' and 'designer babies' as a potential application of the CRISPR/Cas9 technology. The idea that CRISPR sets events in motion that can alter humanity's balance, for better or worse, is a matter best left for a more suitably timed discussion. Events such as these may transpire; such a statement can be acknowledged based on theoretical grounds and educated conjecture. It cannot, however, be ascertained given the current ambiguity in ongoing research. Instead, they nurture an impression whereby this technique is viewed either positively or negatively, especially in light of concerns regarding the ethical implications of germ line alterations. Although posing a legitimate possibility, current experiments have fallen considerably short of making such predictions a reality. CRISPR/Cas9's specificity has yet to be fully ascertained.

Authors' Contributions

Crystal Salinas was responsible for writing and editing the manuscript and contributed to its intellectual conception. Gen Kaneko and Hashimul Ehsan guided the paper's tone and concept, identified areas in need of revision and approved the final form.

Acknowledgements

We would like to thank Zainab Jafor for their insight and helpful remarks during the conception of this paper. We also extend the deepest appreciation to the Arts and Sciences Department at the University of Houston-

Victoria for encouraging students to reach new horizons in scientific knowledge.

Reference

1. Savic, N., and Schwank, G. 2016. Advances in therapeutic CRISPR/Cas9 genome editing. *Translational Research* **168**: 15-21. Doi: 10.1016/j.trsl.2015.09.008.
2. Ishino, Y., Shinagawa, H., Makino, K., Amemura, M. and Nakata, A. 1987. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *J. Bacteriol.* **169**, 5429-5433.
3. Koonin, E.V., and Krupovic, M. 2014. Evolution of adaptive immunity from transposable elements combined with innate immune systems, *Nat. Rev. Genet*. **16**:184e192.
4. Carroll, D., Alta Charo, R. 2015. The societal opportunities and challenges of genome editing. *Genome Biol.* **16**: 242. DOI: 10.1186/s13059-015-0812-0.
5. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. A. 2012. programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**:816–21.
6. Wu, Y., Liang, D., Wang, Y. et al. 2013. Correction of a genetic disease in mouse via use of CRISPR-Cas9. *Cell Stem Cell* **13**:659–62.
7. Long, C., McAnally, J.R., Shelton, J.M., Mireault, A.A., Bassel-Duby, R., and Olson, E.N. 2014. Prevention of muscular dystrophy in mice by CRISPR/ Cas9-mediated editing of germline DNA. *Science* **345**: 1184–8.
8. Yin, H., Xue, W., Chen, S. et al. 2014 Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nat Biotechnol* **32**:551–3.
9. Ding, Q., Strong, A., Patel, K.M. et al. 2014. Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing. *Circ Res* **115**: 488–92.
10. Xie, F., Ye, L., Chang, J.C. et al. 2014. Seamless gene correction of β-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac. *Genome Res* **24**:1526–33.
11. Deng, N., and Chen, Y.J. Application of CRISPR-Cas9 gene editing system: non-viral delivery strategies and improvements. *J Med Discov*. **3**, 7057.
12. Wojtal, D. et al. 2016. Spell checking nature: versatility of CRISPR/Cas9 for developing treatments for inherited disorders. *Am. J. Hum. Genet.* **98**, 90-101.
13. Fogleman, S., Santana, C., Bishop, C., Miller, A., Capco, D.G. 2016. CRISPR/Cas9 and mitochondrial gene replacement therapy: promising techniques and ethical considerations. *Am. J. Stem Cells.* **5**, 39-52.
14. Liang, P. et al. 2015. CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. *Protein Cell.* **6**, 363-372.
15. Wang, S., Yi, F., Qu, J. 2015. Eliminate mitochondrial diseases by gene editing in germ-line cells and embryos. *Protein Cell*. **6**, 472-475.
16. Firth, A.L. et al. 2015. Functional gene correction for cystic fibrosis in lung epithelial cells generted from patient iPSCs. *Cell Rep.* **12**, 1385-1390.
17. Schwank, G. et al. 2013. Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell*. **13**, 653-658.
18. Yang, X. 2015. Applications of CRISPR-Cas9 mediated genome engineering. *Mil. Med. Res.* **2**, 11.
19. Dow, L.E. 2015. Modeling disease *in vivo* with CRISPR/Cas9. *Trends Mol. Med.* **21**, 609-621.
20. Abraham, D.A., Jacobsohn, A., and Bollard, C.M. 2016. Cellular therapy for sickle cell disease. *Cytotherapy.* **18**, 1360-1369.
21. Agrawal, R.K., Patel, R.K., Shah, V., Nainiwal, L., and Trivedi, B. 2014. Hydroxyurea in sickle cell disease: drug review. *Indian J. Hematol. Blood Transfus.* **30**, 91-96.
22. Hoban, M.D. et al. 2016. CRISPR/Cas9-mediated correction of the sickle mutation in human CD34+ cells. *Mol. Ther.* **24**, 1561-1569.
23. DeWitt M.A. et al. 2016. Selection-free genome editing of the sickle cell mutation in human adult hematopoietic stem/progenitor cells. *Sci Transl Med.* **8**, 360ra134. doi:10.1126/scitranslmed.aaf9336.
24. Ye, L., Wang, J., Tan, Y., Beyer, A.I., Xie, F., Muench, M.O. and Kan, Y.W. 2016. Genome editing using CRISPR/Cas9 to create the HPFH genotype in HSPCs: an approach for treating sickle cell disease and β-thalassemia. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 10661-10665.
25. Lettre, G. and Bauer, D.E. 2016. Fetal haemoglobin in sickle-cell disease: from genetic epidemiology to new therapeutic strategies. *Lancet*. **387**, 2554-2564.
26. Hoban, M.D. et al. 2015. Evaluation of TALENs and the CRISPR/Cas9 nuclease system to correct the sickle cell disease mutation. *Mol. Ther.* **23**, S135.
27. Xu, P. et al. 2015. Both TALENs and CRISPR/Cas9 directly target the HBB IVS2-654 (C > T) mutation in β-thalassemia-derived IPSCs. *Sci. Rep.* **5**, Article number: 12065.
28. Finotti, A. et al. 2015. Recent trends in the gene therapy of β-thalassemia. *J. Blood Med.* **6**, 69-85.
29. Xie, F., Ye, L., Chang, J.C., Beyer, A.I., Wang, J., Meunch, M.O., and Kan, Y.W. 2014. Seamless gene correction of β-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and *piggyBac. Genome Res.* **24**, 1526-1533.
30. Yang, Y. et al. 2016. Naïve induced pluripotent stem cells generated from β-thalassemia fibroblasts allow efficient gene correction with CRISPR/Cas9. *Stem Cells Transl. Med.* **5**, 8-19.
31. Liu, Y. *et al*. 2017. One-step biallelic and scarless correction of β-thalassemia mutation in patient-specific iPSCs without drug selection. *Mol. Ther. Nucleic Acids.* **6**, 57-67.
32. Shin, J.W. et al. 2016. Permanent inactivation of Huntington's disease mutation by personalized allele-specific CRISPR/Ca9. *Hum. Mol. Genet.* **25**, 4566-4576.
33. Talan, J. 2015. News from the Society for Neuroscience annual meeting: gene edting techniques show promise in silencing or inhibiting the mutant Huntington's disease gene. *Neurology Today*. **15**, 14-16.
34. Im, W., Moon, J., and Kim, M. 2016. Applications of CRISPR/Cas9 for gene editing in hereditary movement disorders. *J. Mov. Disord.* **9**, 136-143.
35. Xu, X. et al. 2017. Reversal of phenotypic abnormalities of CRISPR/Cas9-mediated gene correction in Huntington disease patient-derived induced pluripotent stem cells. *Stem Cell Reports.* **8**, 619-633.
36. Monteys, A.M., Ebanks, S.A., Keiser, M.S., and Davidson, B.L. 2017. CRISPR/Cas9 editing of the mutant huntingtin allele *in vitro* and *in vivo*. *Mol. Ther.* **25**, 12-23.
37. Nguyen, T.H., and Anegon, I. 2016. Successful correction of hemophilia by CRISPR/Cas9 genome editing *in vivo*: delivery vector and immune responses are the key to success. *EMBO Mol. Med.* **8**, 439-441.
38. Guan, Y. et al. 2016. CRISPR/Cas9-mediated somatic correction of a novel coagulator factor IX gene mutation ameliorates hemophilia in mouse. *EMBO Mol. Med.* 8(5): 477–488. e201506039. doi: 10.15252/emmm.201506039.
39. Park, C.Y., Lee, D.R., Sung, J.J., and Kim, D.W. 2016. Genome-editing technologies for gene correction of hemophilia. *Hum. Genet.* **135**, 977-981.
40. Park, C.Y. et al. 2015. Functional correction of large factor VIII gene chromosomal inversions in hemophilia A patient-derived iPSCs using CRISPR/Cas9. *Cell Stem Cell.* **17**, 213-220.
41. Qihan, W., Cong, H., Ruilin, S., Hua, Z., Hongyan, C., Jian, F., and Daru, L. 2015. A quick and efficient method to generate hemophilia B mouse models by the CRISPR/Cas system. *J. Genet. Genomics.* **37**, 1143-1148.
42. Long, C., McAnally, J.R., Shelton, J.M., Mireault, A.A., Bassel-Duby, R., and Olson, E .N. 2014. Prevention of muscular

dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science.* **345**, 1184-1188.
43. Nelson, C.E. et al. 2016. *In vivo* genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science*. **351**, 403-407.
44. Tabebordbar, M. et al. 2016. *In vivo* gene editing in dystrophic mouse muscle and muscle stem cells. *Science*. **351**, 407-411.
45. Bengtsson, N.E. et al. 2017. Muscle-specific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy. *Nat. Commun.* **8**, 14454.
46. Young, C.S. et al. 2016. A single CRISPR-Cas9 deletion strategy that targets the majority of DMD patients restores dystrophin function in hiPSC-derived muscle cells. *Cell Stem Cell.* **18**, 533-540.
47. Long, C. et al. 2016. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science.* **351**, 400-403.
48. Lattanzi, A. et al. 2017. Correction of the exon 2 duplication in DMD myoblasts by a single CRISPR/Cas9 system. *Mol. Ther. Nucleic Acids.* **7**, 11-19.
49. Veleri, S., Lazar, C.H., Chang, B., Sieving, P.A., Banin, E., and Swaroop, A. 2015. Biology and therapy of inherited retinal degenerative disease: insights from mouse models. *Dis. Model Mech.* **8**, 109-129.
50. Bakondi, B. et al. 2016. *In vivo* CRISPR/Cas9 gene editing corrects retinal dystrophy in the S334ter-3 rat model of autosomal dominant retinitis pigmentosa. *Mol. Ther.* **24**, 556-563.
51. Bassuk, A.G., Zheng, A., Li, Y., Tsang, S.H., and Mahajan, V.B. 2016. Precision medicine: genetic repair of retinitis pigmentosa in patient-derived stem cells. *Sci. Rep.* **6**, 19969.
52. Ruan, G.X., Barry, E., Yu, D., Lukason, M., Cheng, S.H., and Scaria, A. 2017. CRISPR/Cas9-mediated genome editing as a therapeutic approach for leber congenital amaurosis 10. *Mol. Ther.* **25**, 331-341.
53. Latella, M.C., Di Salvo, M.T., Cocchiarella, F., Benati, D., Grisendi, G., Comitato, A., Marigo, V., and Recchia, A. 2016. *In vivo* editing of the human mutant rhodopsin gene by electroporation of plasmid-based CRISPR/Cas9 in the mouse retina. *Mol. Ther. Nucleic Acids.***5**, e389.
54. Wu, W.H. et al. 2016. CRISPR repair reveals causative mutation in a preclinical model of retinitis pigmentosa. *Mol. Ther.* **24**, 1388-1394.
55. Kennedy, E.M. and Cullen, B.R. 2015. Bacterial CRISPR/Cas DNA endonucleases: a revolutionary technology that could dramatically impact viral research and treatment. *Virology*. **479-480**, 213-220.
56. Limsirichai, P., Gaj, T., and Schaffer, D.V. 2016. CRISPR-mediated activation of latent HIV-1 expression. *Mol. Ther.* **24**, 499-507.
57. Lin, A. and Klase, Z. 2016. A CRISPR approach for reactivating latent HIV-1. *Mol. Ther.* **24**, 416-418.
58. Wang, G., Zhao, N., Berkhout, B., and Das, A.T. 2016. CRISPR-Cas9 can inhibit HIV-1 replication but NHEJ repair facilitates virus escape. *Mol. Ther.* **24**, 522-526.
59. Wang, Z., Pan, Q., Gendron, P., Zhu, W., Guo, F., Cen, S., Wainberg, M.A. and C. Liang, C. 2016. CRISPR/Cas9-derived mutations both inhibit HIV-1 replication and accelerate viral escape. *Cell Rep.* **15**, 481-489.
60. Wang, G., Zhao, N., Berkhout, B. and Das, A.T. 2016. A combinatorial CRISPR-Cas9 attack on HIV-1 DNA extinguishes all infectious provirus infected T cell cultures. *Cell Rep.* **17**, 2819-2826.
61. Kang, H.J., Minder, P., Park, M.A., Mesquitta, W.T., Torbett, B.E., and Slukvin, I.I. 2015. CCR5 disruption in induced pluripotent stem cells using CRISPR/Cas9 provides selective resistance of immune cells to CCR5-tropi HIV-1 virus. *Mol. Ther. Nucleic Acids.* **4**, e268.
62. Xi, L., Schmidt, J.C., Zaug, A.J., Ascarrunz, D.R. and Cech, T.R. 2015. A novel two-step genome editing strategy with CRISPR/Cas9 provides new insights into telomerase action and TERT gene expression. *Genome Biol.* **16**, 231.
63. White, M.K., Hu, W. and Khalili, K. 2015. The CRISPR/Cas9 genome editing methodology as a weapon against human viruses. *Discov. Med.* **19**, 255-262.
64. Kennedy, E.M. 2014. Inactivation of the human papillomavirus E6 or E7 gene in cervical carcinoma cells by using a bacterial CRISPR/Cas RNA-guided endonuclease. *J. Virol.* **88**, 11965-11972.
65. Zhen, S., Hua, L., Takahashi, Y., Narita, S., Liu, Y.H. and Li, Y. 2014. *In vitro* and *in vivo* growth suppression of human papillomavirus 16-positive cervical cancer cells by CRISPR?Cas9. *Biochem. Biophys. Res. Commun.* **450**, 1422-1426.
66. Yu, L. et al. 2015. Disruption of human papillomavirus 16 *E6* by clustered regularly interspaced short palindromic repeat/Cas system in human cervical cancer cells. *Onco. Targets Ther.* **8**, 37-44.
67. Hu, Z. et al.2014. Disruption of HPV16-E7 by CRISPR/Cas system induces apoptosis and growth inhibition in HPV16 positive human cervical cancer cells. *Biomed. Res. Int.* **2014**, 612823.
68. Ehrke-Schulz, E. and Ehrhardt, A. 2016. High-capacity adenoviral vectors (HCAdV) armed with a high-risk human papillomavirus (HPV) oncogene specific CRISPR/Cas9 machinery specifically kill cervical cancer cells. *Mol. Ther.* **24**, 32912-32914.
69. Zhen, S., Lu, J.J., Wang, L.J., Sun, X.M., Zhang, J.Q., Li, X., Luo, W.J. and Zhao, L. 2016. *In vitro* and *in vivo* synergistic therapeutic effect of cisplatin with human papillomavirus16 E6/E7 CRISPR/Cas9 on cervical cancer cell line. *Transl. Oncol.* **9**, 498-504.
70. Lin, G., Zhang, K. and Li, J. 2015. Application of CRISPR/Cas9 technology to HBV. *Int. J. Mol. Sci.* **16**, 26077-26086.
71. Ramanan, V. et al. 2015. CRISPR/Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus. *Sci. Rep*. **5**, 10833.
72. Kennedy, E.M. et al. 2015. Suppression of hepatitis B virus DNA accumulation in chronically infected cells using a bacterial CRISPR/Cas RNA-guided DNA endonuclease. *Virology.* **476**, 196-205.
73. Li, H. et al. 2017. Removal of integrated hepatitis B virus DNA using CRISPR-Cas9. *Front. Cell Infect. Microbiol.* **7**, 91.
74. Sakuma, T., Masaki, K., Chayama-Abe, H., Mochida, K., Yamamoto, T. and Chayama, K. 2016. Highly multiplexed CRISPR-Cas9-nuclease and Cas9-nickase vectors for inactivation of hepatitis B virus. *Genes Cells*. **21**, 1253-1262.