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The use of CRISPR/Cas9 gene editing system in the treatment of HBV infection

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Abstract: Hepatitis B disease is an infectious disease caused by the Hepatitis B virus (HBV) which may have different consequences such as cirrhosis and liver cancer. Although there is an effective vaccine against HBV, it is still a serious health problem for underdeveloped and some developing countries where the vaccination rate is low. Current treatments cannot clear all the viral infections. Therefore, there is an urgent need for definitive HBV treatment. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) method is frequently used in vitro and in vivo for genetic modifications, including targeting the HBV genome. Therefore, in this review, the use of CRISPR/Cas9 technology in the HBV genome for treatment purposes will be discussed with the aid of recent literature.

Key words: cccDNA, CRISPR/Cas9, gene editing, HBV

1. Introduction

Despite the intensity of the studies; infectious diseases that are caused by bacteria, viruses, and parasites account for one-quarter of deaths worldwide (Doerflinger, et al. 2017). According to the World Health Organization, more than 240 million people worldwide are chronically infected with HBV. The disease is very common in the Asia and Africa regions. Although the majority of patients do not develop hepatic complaints, 15-40% of these patients develop serious problems related to infection (Gish, et al. 2015). Chronic HBV carriers are at high risk for fatal complications including cirrhosis, end-stage liver disease and hepatocellular carcinoma, resulting in around one million deaths per year (Ramanan, et al. 2015; Yang and Chen 2018). It is reported that liver cancer is located the third place in cancer-related deaths (Moyo, et al. 2018).

HBV is still a significant human pathogen, although there is a greatly effective vaccine against it. Vaccination rates continue to remain below 100%, especially in countries with limited resources, and although infants born from HBV positive mothers are vaccinated at birth, this is not fully protective against vertical transmission (Komatsu 2014).

Recently, the CRISPR/Cas9 has been utilized for various genetic regulations both in vitro and in vivo experiments (Platt, et al. 2014). A CRISPR/Cas9 system has been used for many purposes such as protective and therapeutic (Xiao-Jie, et al. 2015). Finding treatment against infectious diseases including HBV is one of the most significant goals of CRISPR/Cas9 application. For this reason, in this short review, HBV, CRISPR/Cas9 system and its applications in HBV will be discussed.

1.1. HBV Structure and Disease

HBV belongs to the Hepadnaviridae family that is the hepatotropic DNA viruses, and based on the nucleotide sequence of HBV eight genotypes (A-H) are known (Karayiannis 2017). The HBV consists of a viral membrane covered with surface proteins. The membrane surrounds a core particle that contains the viral DNA genome of the multi-functional HBV polymerase (Karayiannis 2017).

The HBV virus genome is depicted in Figure 1. The enveloped virus HBV has a partially double-stranded DNA about 3.2 kb size (Tsai, et al. 2018). This DNA also called covalently closed circular DNA (cccDNA) that is the crucial element of the HBV life cycle (Ramanan, et al. 2015). It functions as a mini-chromosome within the nucleus, serving as a template for cellular enzymes to synthesis of new viral pre-genomic and sub-genomic messenger RNA (Pollicino, et al. 2014). Flap structure-specific endonuclease 1 (FEN1) is involved in HBV cccDNA formation (Kitamura, et al. 2018). The genome of HBV consist of four open reading frames (ORFs) called preS1/preS2/S, preCore/Core, X and Pol that are translated into viral core protein, surface proteins, polymerase/reverse transcriptase (RT), and HBx (Pollicino, et al. 2014) (Song, et al. 2018).

The three different and structurally related viral surface antigens are encoded by the ORF of PreS1/preS2/S. The envelope glycoproteins collectively recognized as HBV surface antigen (HBsAg) include large (LHBs), medium (MHBs), and small (SHBs) surface proteins that are important for HBV-positive hepatocellular carcinoma (Glebe 2007; Song, et al. 2018).

DNA polymerase is encoded by P genes and the last ORF; X encodes HBx protein that has many different functions in HBV development (Tsai, et al. 2018). Evidence shows that the several gene products of HBV have been accepted as viral oncoproteins. For example, HBx protein could interfere with cell signaling and transcription of genes and may have influence on cell growth,

cell cycle and HCC metastasis (Casciano and Bouchard 2018; Lamontagne, et al. 2018; Slagle and Bouchard 2018).

Chronic production of HBV antigens may lead to inflammation and necrosis. This may cause liver enzymes elevation, hepatitis, cirrhosis, hepatocellular carcinoma, and liver failure (Li, et al. 2018). In liver cells, HBV pathogenicity is primarily related to cell-mediated immune response. While HBsAg and HBeAg cause hepatitis and raise of transaminase, HBcAg activates the CD8 response of cytotoxic T lymphocytes and consequently end with liver cells damage. Persistence of chronic hepatitis and cirrhosis causes liver cancer and liver failure (Wooddell, et al. 2013). The initiation of hepatitis and cirrhosis could be successfully inhibited by decreasing serum HBcAg, HBsAg, and HBeAg levels in liver cells (Wooddell, et al. 2013).

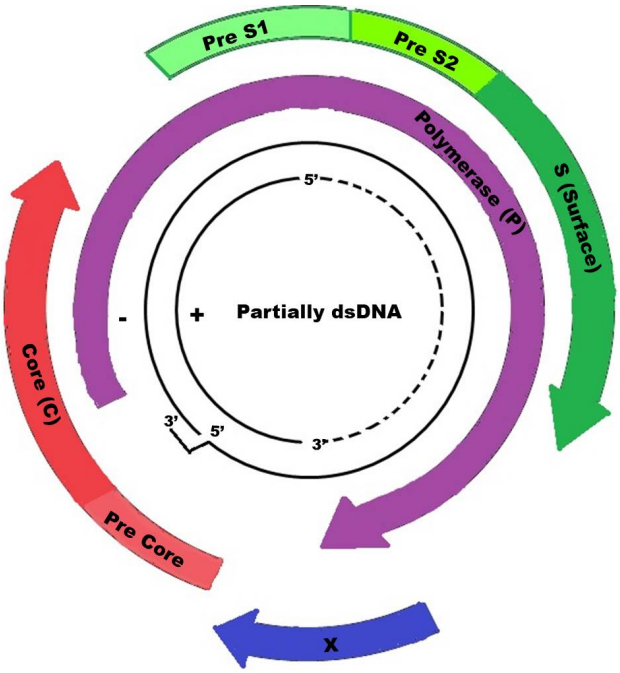

Figure 1. Hepatitis B virus (HBV) Genome (Re-drawn from Bell and Kramvis) (Bell and Kramvis 2016) The core proteins are encoded by C genes. HBV core proteins that controls host gene synthesis, like hFGL2 and p53, influence the biological activity of hepatocytes (Song, et al. 2018).

1.2. Treatment of HBV

Given the heavy burden caused by chronic hepatitis B, chronic HBV treatment is still an urgent medical need for universal public health. Vaccination is one of the greatest achievements of research in the field of infectious diseases and millions of people are protected from bacterial and viral infections each year by vaccination. However, expensive vaccine production, accession to primary health care facilities, and problems that prevent the distribution and maintenance of the cold-chain are important barriers for universal vaccination (Doerflinger, et al. 2017). Although HBV vaccine protects infants from infection, HBV carriage in some regions of sub-Saharan Africa and East Asia varies between 5-10%. This carrier statistic also increases the number of individuals infected with HBV. Therefore, HBV is an important health problem all over the world (Dong, et al. 2015)

The treatment of HBV is mainly based on the use of nucleoside analog chain terminators including tenofovir disoproxil fumarate (TDF), lamivudine (3TC), and entecavir (ETV). They are worked by inhibiting HBV reverse transcriptase (RT) activity (Kostyusheva, et al. 2018).

Although using HBV-RT inhibitors clearly slow the development of cirrhosis and HCC, they cannot completely eliminate the viral infection. This is due to the highly stable cccDNAs that persist in the nucleus of infected hepatocytes and serve as templates for viral mRNA and pre-genomic RNA synthesis (Werle-Lapostolle, et al. 2004). Although RT inhibitors could stop de-novo HBV infection of hepatocytes, infected cells remain infected during treatment initiation and are only slowly reduced due to cell turnover (Kennedy, et al. 2015). Besides, even after years of treatment, latent, non-replicating viral genomes remain in reservoirs and typically maintain elevation of viral replication immediately after discontinuation of antiviral therapy (Hongthanakorn, et al. 2011). Therefore, lifelong treatment of viral diseases is often necessary, which results in huge costs for health care, and sometimes these long-term treatments result in simultaneous resistance e.g. HIV (Trono, et al. 2010; Zoulim 2011). In addition, treatment due to lack of compatibility, drug toxicity, and resistance may also become increasingly complex (Schiffer, et al. 2013). Interferon-alpha (IFN-α) HBV therapies in some patients may clear HBV DNA but it cannot be tolerated because of the side effects of high-dose or long-term treatment (Ohno, et al. 2015; Wursthorn, et al. 2006). Current HBV antivirals and vaccination, which are used in the treatment and prevention of HBV, are beneficial for stopping infection and inhibiting viral replication, but existing treatments cannot offer a definitive or functional treatment for HBV infection (Doerflinger, et al. 2017). Cope with this challenge is one of the most important aims of HBV research (Kurihara, et al. 2017).

To ensure complete HBV treatment, either all infected hepatocytes should be eliminated or persistent intrahepatic cccDNAs must be cleared (Lin, et al. 2016). A second strategy is the specific destruction of the HBV genome without damaging the host genomes (Yang and Chen 2018). This is now possible with programmable RNA-driven DNA endonucleases derived from the CRISPR/Cas9 mechanism (Hsu, et al. 2014).

1.3. CRISPR/Cas9 Technology

Genetic engineering, manipulation of DNA or RNA, is being used at an increasing rate to stop or treat diseases (Pickar-Oliver and Gersbach 2019). The most common methods used for target-specific gene regulation are zinc finger nuclease (ZFNs), transcription activator-like effector nuclease (TALENs) and most recently the CRISPR/Cas system. ZFNs and TALENs are meganuclease proteins that are capable of recognizing specific DNA sequences and are guided by proteins (Kim and Kim 2014). In contrast to meganucleases, CRISPR/Cas technology is RNA guided system that target sequences bind to single guide RNA (sgRNA) (Mali, et al. 2013).

The CRISPR system is firstly described in Archaea and Bacteria. It inhibits the spread of plasmids and viruses to these organisms by RNA-induced adaptive immune system defense (Memi, et al.

2018). Different types of Cas proteins are available but the most commonly used one is Cas9 nuclease that belongs to the type II CRISPR system and encodes a protein with multi-domain that integrates entire activities of effector complexes and cleavage of the target DNA. An RNA molecule called guide RNA (gRNA) directs Cas9 and forms a direct link to the target DNA by Watson-Crick base pairing, causes DNA double-strand breaks (DSBs) (Makarova and Koonin 2015). The host cell responds to these DSBs in two different ways: non-homologues end joining (NHEJ) and homology-directed repair (HDR). While NHEJ leads to an insertion-deletion and a frameshift mutation in the target DNA, donor template is used for homologous recombination in HDR. By this mechanism, after DSB, the donor template is used for DNA repairing instead of NHEJ pathway that helps precise genome editing. (Salsman and Dellaire 2017). There are wide range of applications of Cas9 in genetic engineering including gene regulation, gene expression, and gene function. Cas9 has received great consideration in the treatment of various diseases initiated by mutations and infection including viral infections and cancers (Jia 2018).

CRISPR/Cas9 systems are more advantageous than ZFN or TALEN based gene editing strategies and have many significant advantages over them including:

1) ZFN and TALEN require more effort and are more expensive than CRISPR/Cas9 technology (Doudna and Charpentier 2014).
2) For the administration of the CRISPR/Cas9 system, Cas9 nuclease protein is the same in each case and only 20-base pairs of sgRNAs need to be identified (gRNAs, however, needs to be designed individually); nonetheless, meganuclease should be generated for each case individually on ZFN or TALEN-based strategies (Xiao-Jie, et al. 2015).
3) Finally, unlike ZFN and TALEN, CRISPR/Cas9 systems have the potential for simultaneous amplification or suppression of the target genes. Therefore, the simplicity and the possibility of improving Cas9 proteins are the main factors that allow the CRISPR/Cas9 system to be widely used when developing new drugs (He, et al. 2016).

Despite its effectiveness in genome editing, the CRISPR/Cas9 system has a number of challenges to consider, such as gRNA production and efficient delivery, but the major concern of this technique is the genome editing because of the non-specific activity of Cas9 (off-target effect) that causes undesirable mutations and mosaicism, which is a result of early cells division previous to genome editing (Wang, et al. 2016). In somatic cell cultures, the extraction of correct target clones in both cases (off-target and mosaicism) is simpler, whereas CRISPR is more difficult when applied to germ-line cells (Cho, et al. 2014).

To escape off-target effects and to guarantee the specificity of CRISPR/Cas9 efficiency, it is better to choose target sequences with the minimum homology to off-target regions. Besides, the dose of CRISPR/Cas9 is another factor that influences target effects and needs attention (Zhang, et al. 2014). The gRNA structure and composition of influence the off-target effects, as well. Since CRISPR/Cas9 leads to permanent changes in cells, off-target effects should be cautiously managed.

In spite of many effective uses of CRISPR/Cas9, there are some restrictions in clinical practice. First, it is the immunogenicity of viral vectors such as adenovirus and lentivirus used for efficient Cas9-gRNA therapeutic delivery (Schumann, et al. 2015). In addition, the use of lentiviral transduction for Cas9-gRNA distribution may cause insertion mutagenesis, which ultimately results in the silencing of several other genes. To solve these problems, less immunogenic AAV or the integrase-deficient lentivirus vector is used (Liu 2014). Using some orthologs such as SaCas9 or using Split-Cas9 system can also solve this packaging problem (Ran, et al. 2015). The risk of off-target in non-infected tissues will also increase the systemic effect of antiviral CRISPR/Cas9 (Yin, et al. 2016)

1.4. CRISPR/Cas9 Applications in HBV Treatment

Permanent treatment of viral infections is possible by targeting non-replicating viral genomes. DNA endonucleases, including homing endonucleases (HE) or meganucleases, ZFN, TALENs, and Cas9 proteins, are promising new therapies to target these viral forms. DNA endonucleases could be used to target specific episomal DNA segments that are vital for HBV replication (Aubert, et al. 2011; Cradick, et al. 2010; Kennedy, et al. 2015; Seeger and Sohn 2014). Furthermore, gene regulation therapy with CRISPR/Cas9 technology has already been tried in clinical trials in patients with HIV, leukemia or solid cancer (Tebas, et al. 2014).

After viral DNA is cleaved by endonucleases, it is rapidly repaired and allows the cleavage enzyme to bind repeatedly. If there is no mutation during repair, the enzyme binds to the target site again, but eventually, there is deletion or insertion mutation that prevents the translation of essential viral proteins as well as subsequent enzyme binding in the target DNA. Ultimately, the remaining mutant viral DNA becomes insufficient for replication (Schiffer, et al. 2013).

According to a previously developed mathematical model that defined the delivery and intracellular activity of DNA cleavage enzymes, it was predicted that the use of a high amount of vector relative to the target cell, restricted elimination of delivery vectors from humoral immunity, and higher binding power between enzyme and target DNA would increase cccDNA degradation level (Schiffer, et al. 2013). De-novo cleavage enzymes resistance may develop if DNA damage and error-prone repair do not render viral episome replication inadequate. Simultaneous or sequential administration of multiple enzymes targeting different regions of vital cccDNA is potentially useful strategies to avoid multiple enzyme resistance. According to this model, the dynamics underlying cccDNA persistence, simultaneous administration of antiviral therapy during eradication trials are predicted to not affect the likelihood of recovery (Schiffer, et al. 2013).

The distinctive benefits of the CRISPR/Cas9 technology, like multiple targeting, are of interest in the development of antiviral approaches. Many researchers have recently published studies related to the cleavage of HBV with Cas9 in various model

systems (Kennedy, et al. 2015; Lin, et al. 2014; Liu, et al. 2015; Peng, et al. 2015; Zhen, et al. 2015).

Zhu et al. generated two homologous sequences (S and X genes) of HBV-specific CRISPR/Cas9 systems. They have reported that pCas9 targeting X genes produce better anti-HBV effects in the in vitro and in vivo model (Zhu, et al. 2016). gRNA targeting the HBV surface antigens and DNA polymerase encoding regions could inhibit viral replication efficiently with minimum off-target effects and less influence on cell viability (Li, et al. 2016).

Although Cas9-sgRNAs can lead to the desired mutations on the target DNA, the possibility of undesired off-target mutations in the host genome is high (Pattanayak, et al. 2013). To enhance specificity on the targeted DNA and decrease unwanted off-target mutations in the host genome, an approach has been developed using nickase-Cas9 to neutralize any of the RuvC and NHN nuclease domains of Cas9 (Shen, et al. 2014). Nickase-Cas9 only cuts a single strand of the target DNA. Therefore, pair of sgRNAs targeting both DNA strands is needed to stimulate DSBs on the target DNA. This increases Cas9 cleavage specificity in the host genome and reduces the off-target effects. In such a study, nickase-Cas9 with two sgRNAs targeting the HBV genome has been shown to provide efficient cleavage and suppress HBV replication. Remarkably, nuclease dead Cas9 (d-Cas9) has also been shown to suppress replication of HBV similarly without cleavage of HBV genome (Kurihara, et al. 2017). Targeted disruption of S and X gene of HBV with Cas9 nickase leads to not only episomal cccDNA and chromosomally integrated HBV target sites disruption in reporter cell lines, but also disruption of HBV replication in chronically and de novo infected hepatoma cell lines, similar results were obtained in Wang et.al. study (Karimova, et al. 2015; Wang, et al. 2015). Targeting three critical areas of the HBV genome at the same time with highly multiplexed CRISPR/Cas9 nuclease and Cas9-nickase vector lead to markedly fragmented HBV genome, minimal off-target effect, and a significant decrease in extracellular hepatitis B surface antigens, envelope antigens and viral replicative intermediates level (Sakuma, et al. 2016).

The activity of CRISPR/Cas9 destroying HBV DNA was also investigated in a modified NHEJ/HR environment. NU7026, a potent inhibitor of NHEJ, prevent the degradation of CRISPR/Cas9 mediated cccDNA and caused deletions on the target. Therefore it is argued that CRISPR/Cas9 is a very powerful tool for cccDNA degradation, whereas inhibition of the NHEJ pathway inhibits cccDNA degradation (Kostyushev, et al. 2019)

In addition to the CRISPR/Cas9 system itself, there are other studies combining CRISPR/Cas systems with different molecules or inhibitory systems. For example, APOBEC, that leads to the deamination of cytosine residues on the minus strand of HBV cccDNA and recommended for HBV treatment, but the effectiveness on cccDNA degradation is much less than CRISPR/Cas9 (Seeger and Sohn 2016). Additionally, CRISPR/Cas9 and RNAi combination have a synergistic effect in suppressing HBV replication and destruction not only the HBV genome but also cccDNA in vitro and in vivo models (Wang, et al. 2017). The effect of sgRNAs targeting the S and P regions in the duck HBV (DHBV) genome was also investigated using the CRISPR/ Cas9 system and entavir (ETV) anti-viral effects. The CRISPR/Cas9 system alone inhibits DHBV total DNA and cccDNA. The combining of CRISPR/Cas9 and ETV has been shown to induce the suppression of DHBV total DNA but does not alter cccDNA (Zheng, et al. 2017). In another study, two different small molecules were used together with the CRISPR/Cas9 system that effectively inhibited the transcription and replication of HBV. These two small molecular compounds are RI-1, that inhibits the binding of the filament RAD51 structure and the HDR pathway, and NU7026, which irreversibly binds to the catalytic subunit of the DNA protein kinase (DNA-PKcs) (Kostyusheva, et al. 2019). It has been shown that the administration of these two molecules to cells separately and together is not toxic. In addition, the treatment of HBV-infected cells with NU7026 and RI-1 has been shown to elevate the antiviral effect of the CRISPR/Cas9 system and reduce cccDNA levels by 70-89% and 58-94%, respectively. However, these two components did not cause synergistic effects when administered together (47-74%) (Kostyusheva, et al. 2019).

CRISPR/Cas9 protein is delivered to cells in two different ways including viral and non-viral delivery (physical delivery system). The effectiveness of a delivery method depends on the target cell types and the type of target tissues (Zhang, et al. 2014). The CRISPR/Cas9 system containing three gRNAs is successfully delivered into the cells by using a High-capacity adenoviral vector (HCAd) and its antiviral effect demonstrated (Schiwon, et al. 2018). Serum HBeAg, HBsAg levels, HBV DNA and liver cell HBcAg levels could be reduced without a significant off-target effect in chronic HBV transgenic mice with clear HBV expression by using rAAV8-CRISPR-SaCas9 (Li, et al. 2018; Liu, et al. 2018). Moreover, incorporation of the SaCas9 and sgRNAs encoding cassettes into ssAAVs and targeting the HBV-S region caused to effective inhibition of HBV replication and mutagenesis of cccDNA in cultured cells (Scott, et al. 2017).

In a study using four different types of CRISPR/Cas9 systems including SpCas9, StCas9, NmCas9 and FnCas9, the CRISPR-StCas9 was reported to be a perfect candidate for the improvement of HBV treatment. The reasons for this are as follows: 1) targeting three highly conserved regions in the HBV genome and causing degradation of HBV cccDNA, 2) showing little acceptance to mismatched nucleotide yet targeting single nucleotide variants of HBV, 3) a small number of off-targets in the human genome and 4) no off-target nucleolytic activity (Kostyushev, et al. 2019).

Although it is not reported in HBV studies, the viral escape problem with CRISPR / Cas9 is described in the Pseudo Rabies Herpes virus (Peng, et al. 2016). CRISPR/Cas9 causes the DSB in the virus DNA and silences these breaks causing mutation in the virus DNA after repair by NHEJ. However, some viral subtypes are escaping, surviving and not being recognized by gRNA. Escaping mutant form of viruses evolves by deletion, insertion, and substitution effects on the target sites of Cas9 (Wang, et al. 2016). It could be possible to prevent the formation of escaping mutants by targeting the viral genome at several sites.

2. Conclusions

Many studies have uncovered the enormous effects of CRISPR/Cas9, yet there is still much to learn about the system itself to entirely exploit the power of CRISPR/Cas9 as a great method for fighting against viral diseases. Before clinical application of this system, more caution and deep knowledge are necessary; since, there are some limitations of these studies including the lack of evaluation of long-term inhibitive effects, insufficient broad examination of immune response of host etc.

Finally, although it is currently not possible to definitively treat HBV with the CRISPR/Cas9 system; the CRISPR/Cas9 method is still thought to be a potential new treatment strategy for various malignancies.

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Gene editing studies for the treatment of AIDS

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Abstract: Following after the fatal genetic diseases that were caused by single nucleotide polymorphisms (SNPs) and cancer, HIV/AIDS had always been one of the primary targets of cell and gene therapies due to lack of any proper satisfactory treatment. Earlier gene therapy approaches were mostly trials about introducing anti-HIV genes to cells, using various viral vectors. These viral vectors performed integrations of the desired anti-HIV genes, sometimes correctly while sometimes between random wrong sequences. However, with the increased precision of new gene editing technologies, including ZFNs and the latest CRISPR-mediated gene editing systems (Clustered Regularly Interspaced Short Palindromic Repeats) more successful therapies have begun to be administrated. As an important example of therapy, the trial of Timothy Ray Brown which was followed by the "London patient", allowed the topic of gene editing techniques for treatment of HIV and AIDS to gain interest again.

Key words: AIDS, CRISPR/Cas9, gene editing, HIV

1. Introduction

Human immunodeficiency virus (HIV) has 2 subtypes of HIV-1 and HIV-2, both of which infect some primates, along with humans. Both strains of HIV are thought to be originated from non-human primates and at some time spread to humans. In primates, another virus, very similar to HIV strains called simian inefficiency virus (SIV), can also be present that is thought to be where HIV originated (Sharp and Hahn 2010). In humans both strains of HIV are most likely to cause a deadly condition called "acquired immunodeficiency syndrome" (AIDS) (Bowers, et al. 2014) although HIV-2 strain is less pathogenic and transmissible. AIDS is a deadly condition where the immune system in humans starts to fail and as a result, individuals suffering from AIDS live about 10 years after being infected. While the condition itself doesn't cause death of the individuals the weakened immune system and especially the diminishing number of killer T cells which only keep decreasing due to AIDS makes it impossible for the human system to fight off harmful microorganisms and destroy cancer cells. Cancer cells continuously keep forming in both human and other multicellular biological beings' cells however the immune system identifies and kills these differentiated cells. While some cancer cells do not get immortalized and eventually die due to their senescence even if the immune system does not notice them, immortalized cancerous cells and tissues will have to be dealt with by the organism itself. With the ever-decreasing T cells the body cannot even be able to keep up with rather weak and normally non-problematic infestations. After about 10 years due to failure of infected organs or other tissues and increasing cancerous cells the patients die. However AIDS and the HIV is not lethal all the time as there are some people who are resistant to some strains of HIV and with the aid of gene therapy, the "Berlin patient", Timothy Ray Brown was able to be cured and became naturally resistant to HIV (Brown 2015), becoming the first person to be cured of HIV. Following Brown, eleven years later another patient, who is called the "London patient" (Peluso, et al. 2019; Saez-Cirion and Müller-Trutwin 2019) due to patient's request to remain anonymous, was cured of HIV after being treated in the same method, which will be explained throughout the paper. After these two successful cases of treatment of AIDS via gene therapy, we believe that gene editing's huge potential is only just being noticed. Considering how gene therapy is still an emerging science, it is a future candidate to cure AIDS very easily. Gene therapy can turn HIV into another once deadly disease that used to plague humankind, but now being of no concern anymore like how simple vaccinations are eradicating once terrible deadly diseases like polio or chickenpox.

With a 9.8 kb genome, HIV virus is a retrovirus, which is known for its unique property of carrying RNA as a hereditary genome while all the other virus types carry a single or double DNA strand to express their genome. As retroviruses include RNA based genome instead of DNA, they are also required a special protein called "reverse transcriptase" which is again unique to retroviruses and is used by scientists in the lab for experiments often. HIV is also classified as a lentivirus which is a subtype of retroviruses that is more infectious than regular retroviruses as only cells that are actively dividing are targeted by retroviruses. However, lentiviruses can target cells that are both mitotically active and cells that are not proliferating anymore. While this aspect of lentiviruses makes them highly dangerous it also makes them a lot useful for scientists to take advantage of. When gene therapy experiments are conducted the higher delivery rate of lentiviruses decrease the additional steps for increasing the lentiviral vectors' tropism.

HIV-1 can be transmitted via genital sexual intercourse, intravenous injections like re-using a syringe and vertical transmission during birth (Hladik and McElrath 2008). Body fluids like blood, semen, vaginal fluids and breast milk can carry HIV-1 (Liuzzi, et al. 1996). Acute HIV infection (Cohen, et al. 2011), followed by chronic HIV infection (clinical latency) and then clinical disease (AIDS) are the three stages that HIV-1 infected patients go through in order (Sharp and Hahn 2011). On the surface of the infected cell's membrane the CD4 receptor is bound by gp120 envelope protein of HIV-1 at first, then depending on the virus' tropism it will interact with either CXCR4 co-receptor or CCR5 co-receptor and penetrate into the cell. After HIV manages to get inside the cell, latent and active infections will begin. Infected cells start producing new progeny due to the viral particles produced by provirus during active infection. Chromatin environments (Gallastegui, et al. 2011), transcription factors (Lenasi, et al. 2008), RNA interference (RNAi) (Patel, et al. 2014; Ruelas, et al. 2015) and HIV-1 provirus integration sites (Sunshine, et al. 2016) are complex mechanisms that mediate latent infection establishment. Although, HIV strains infect numerous types of cells their main targets are T cells, monocytes and dendritic cells while they also infect astrocytes, microglial cells and perivascular macrophages of central nervous system (CNS). The complexity of the HIV-1's infectious lifecycle makes its elimination quite hard and complicated, requiring methods like "shock and kill" to achieve a complete eradication. The latent virus reservoirs are hard to reach as they are often located in brain (Fischer-Smith, et al. 2001), gastrointestinal tracts (Smith, et al. 2003) or lymphoid tissues (Chun, et al. 2008) and antiviral drugs can hardly reach such tissues. The latent reservoirs will begin to produce new viruses like actively infected cells once they are stimulated so cleaning these reservoirs is a primary goal of curing HIV/AIDS.

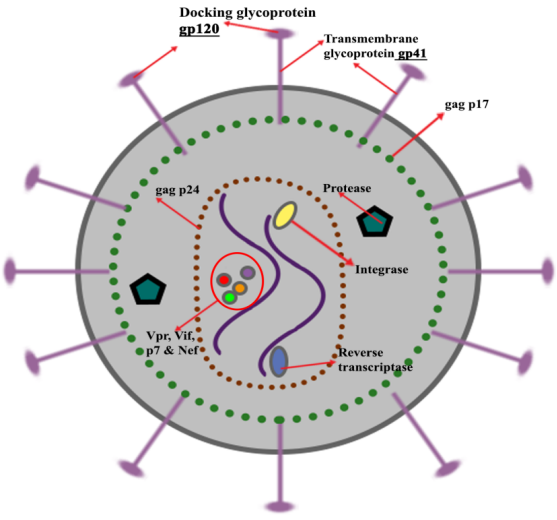

Figure 1. The lentivirus HIV carries docks to host cells via its glycoprotein gp120. To infect T cells, HIV binds to CD4 receptor and CCR5 or CXCR4 co-receptors with gp120.

Along with fatal genetic disorders like cystic fibrosis, SCID or other hereditary diseases that are caused due to SNPs, and cancer, HIV/AIDS was a target of gene editing treatments from the very beginning (Burnett, et al. 2012), (Mylvaganam, et al. 2015). With the recent developing CRIPSR technologies and Brown's case, the HIV/AIDS treatment methods are going under reviews to find a way of curing it in a few steps despite HIV/AIDS being very complex and full of hurdles and setbacks. Protecting the $CD4^+$ T cell from infection via anti-HIV genes constituted most of the gene therapy treatments for HIV up until now. As the genetically engineered cells' selective survival increases the treatment's effectiveness over time, the total viremia was expected to be reduced by decreasing the susceptible cells' number. After the immune functions were restored thanks to $CD4^+$ T cells' direct protection, the body would be able to resist both the HIV and the infection's related symptoms.

Highly active antiretroviral therapies (HAARTs) or also called as antiretroviral drug therapies (ARTs), was introduced in 1990s for treatment of HIV and despite revolutionizing the methods of HIV treatments and drugs, it did not produce a complete HIV eradication. Along with additional harmful effects, people who go through ARTs treatment for several years cannot achieve complete immune response recovery and show elevated levels of immune activation even if they were treated successfully. As HIV can integrate its genome into the cells, becoming a permanent part of the genome, they can have suppressed expressions that will make ART treatments inefficient due to its latent viral nature. Additionally, discontinuation of ART might lead to viremia's rebound due to changes at activation status of cells (Siliciano, et al. 2003).

Mucosa associated lymphoid tissue's (MALT) disruption which is followed by mucosal barrier's disruption along with the ongoing low scale viral replications are assumed to be the main reasons for hyper-activation of immune system in HIV infections, which are now referred to as "immunosenesecence" or "immune aging" (Brenchley, et al. 2006), (Palmer, et al. 2008). Therapeutic anti-retroviral combination strategies are only partially able to correct this. Modern approaches of antiretroviral therapy's significant drawbacks are preferential infection that is followed by CCR5 co-receptor expressing HIV specific T-helper cells getting eliminated. Accumulation of cells that have been latently infected or a low level but persistent viral replication might occur which is undesirable as it can hinder HIV eradication attempts that work by using antiretroviral small molecule drug methods only (Finzi, et al. 1997). Additional ARTs may be used if continuous viral replication cycles and new cells getting infected cause a residual viremia. As mentioned before, cells that have been infected latently can act as virion releasing stable reservoirs which again might cause viremia (Finzi, et al. 1999) and as a result ARTs will have to be applied through patient's whole life which cannot actually cure them. If such a case is present intensification will not be likely to provide results as no viral replication's full cycle is needed for release of HIV. Instead of infections that are latent, the present antiretroviral medications that are approved only target active viral replication enzymes. Living with regular ART routines is not easy, side effects, its accessibility and emotional hardships are challenging. A complete virologic control cannot be achieved by a major portion of patients on ART and its accessibility, as mentioned, is hard as only one third of the total estimated 1.2

million patients with HIV-1 achieve the required ART in a country like United States.

Nowadays new cancer medications are aiming to eradicate HIV via CCR5 antagonists and inhibition of integrases as their mode of working (Murray, et al. 2007; van Lunzen 2007). Although immune activation could be decreased or persistent viral load might be reduced with these strategies, no viral-mediated immune activation's downregulation had been clinically shown by reported intensification trials of HAART (McMahon, et al. 2010; Yukl, et al. 2010). As a result, recovering the immunological properties that were lost shortly after the infection of HIV, is a plausible target for gene therapy approaches along with latent infections and residual replications. Throughout the infection's initial acute phase, the amount of $CD4^+$ T cells decrease which can hamper with the patient's immune system even when the HIV infection is suppressed to full extent. This can be observed from various patients who, despite having an active control over their virological state, were unable to obtain their normal levels of $CD4^+$ T cells.

1.1. Cell/gene therapy and gene editing

A wide range of various vectors and delivery methods have been used that involved all sorts of blood cell types such as cytotoxic T cells (T_c), T-helper cells (T_h) or peripheral blood stem cells. Skewed maturation of T_c augmentations or autologous T_c adoptive transfers were unable to be beneficial in early clinical trials (Walker, et al. 1993). Peripheral T_h were targeted for gene modifications in some other clinical trials (Van Lunzen, et al. 2007).

Including viral RNA decoys (RRE and TAR) (Li, et al. 2005), dominant negative viral proteins (Rev M10) and peptides (C46) and RNA-based methods to block either host or viral genes using antisense RNA (asRNA), ribozymes (Mitsuyasu, et al. 2009) and RNAi, multiple anti-HIV genes were tested in clinical trials (Morgan, et al. 2005). Either $CD4^+$ T cells are targeted ex vivo with the candidate anti-HIV factors or the hematopoietic stem and progenitor cells (HSPC), which are the in vivo precursors of $CD4^+$ T cells. As mentioned retroviral and lentiviral vectors are good vector candidates that are used often in gene therapy as they are especially good at integrating their genome permanently so they are chosen to alter genomes of hematopoietic cells permanently. However just like all the other viral vectors, retroviral and lentiviral vectors have their disadvantages too, such as genotoxicity (Trobridge 2011) by random gene integration or failing to produce a long term gene expression. Although the anti-HIV genes suggested for gene therapy had managed to show their safety and created more interest for future applications, they have not been so successful at being efficient enough (Wang and Cannon 2016).

In the recent years the way of gene editing and gene therapy has shifted towards CRISPR usage where in simple and general principle, specially built nucleases create double strand breaks (DSB) at desired locations and by exploiting the DNA repair mechanisms including non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Lin, et al. 2014) the genes are edited. The provided template is used by the cell while repairing the genome and the genes are edited. NHEJ repair pathway is likely to introduce random insertions or deletions (in/del or indel) to the cut site but using a HDR will produce more specific and "controlled" mutations and gene editing. A single cut on a single strand of double stranded DNA is regarded as a nick while the enzymes that produce nicks by cutting in described manner are called nickases. Aside from specially designed Cas (CRISPR associated protein) that cut by double nickase activity, transcription activator-like effector nucleases (TALEN) or zinc finger nucleases (ZFN) can be used to produce a DSB. However, the method of interest in gene editing is highly shifted to using CRISPR/Cas9 and its variations (Gaj, et al. 2013). The possible genetic changes available with NHEJ are disruption of genes, which are CXCR4 or CCR5 co-receptors or an integrated HIV genome. HDR however, can be used for both adding genes like mentioned genes of anti-HIV or editing the genes such as factors of dependency or restriction.

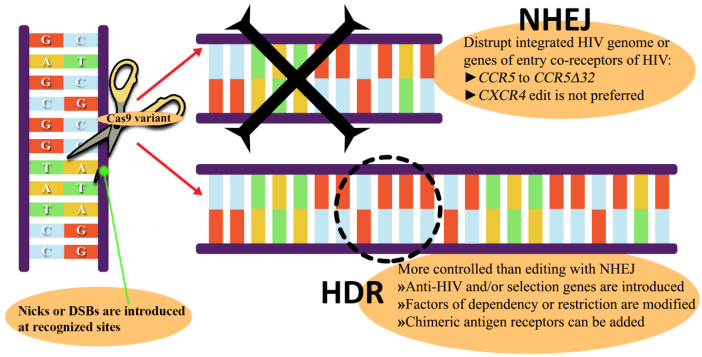

Figure 2. The gene edits are introduced by manipulating the repair mechanisms of DNA in cells. The provided gRNA scaffolds direct how the repair is done by acting as a template.

1.2. Timothy ray brown and CCR5 disruption

A few years after being identified as HIV positive and starting taking low doses of zidovudine (ZDV), which is also known as azido thymidine (AZT), a retroviral drug for AIDS, Brown was diagnosed with leukemia and he went through several treatment procedures targeting his leukemia. Gene editing that is focused on *CCR5* gene to prevent its expression being one of the first clinical applications was not a coincidence, it was chosen specifically as a great target (Lopalco 2010). Disrupting a gene is a lot easier than editing it correctly so using a NHEJ, is a simpler choice that is more likely to be successful in practice. In addition, NHEJ is active during all of the cell cycle unlike HDR, which is largely restricted to G2 and S stages since sister chromatids are able to provide the repair mechanism during that time (Karanam, et al. 2012). Furthermore, some studies had even showed that absence of *CCR5* provides enhanced intelligence in mice (Zhou, et al. 2016) so such a scenario might also be possible in humans with this gene editing, like Brown who went through it or the Chinese twin daughters that were gene edited via CRISPR by He Jiankui, which caused extreme repercussions (Kuersten and Wexler 2019), (Cyranoski and Ledford 2018) and controversies (Li, et al. 2019). Some recently published study even claimed that the gene editing made by He Jiankui is deleterious and creates a shorter life expectancy (Wei and Nielsen 2019), however, the mentioned publication was later withdrawn. Aside from the ethical concerns, the gene editing in the children was still unnecessary as cesarean section birth can protect the babies from HIV and *CCR5Δ32* still only protects

from HIV-1 strains which still does not a guarantee a protection as virus can access CXCR4 as an alternative co-receptor.

After it was decided that Brown was to go under a stem cell transplant from a matching human leukocyte antigen (HLA), out of possible donor choices, Brown's doctor decided to choose a donor with *CCR5Δ32* mutation on their CD4 cells. Brown already had 267 matching donors, which was a huge number so his doctor had the chance of finding a donor with this trait. *CCR5* gene is a good choice for targeting for gene disruption as it is not an essential human gene so while lacking it might grant protection from HIV its presence will not be providing any easily noticed traits although it has known benefits (Glass, et al. 2005). However, the curative effects of HIV/AIDS are favored compared to *CCR5*'s normal functions. Especially being used by early infecting and transmitting strains, most of the strains of HIV use CCR5 protein as an entry co-receptor (Hoffmann 2007). Upon the expression of *CCR5Δ32* allele, which consists of 32 bp deletion hence the name Δ32, the protein that is normally present on the surface of the cells are not created. It was shown by various studies that when this allele is present in homozygous manner, which is the case for about 1% of Caucasian race (Samson, et al. 1996), it grants a protection against HIV-1 strain infection without any adverse side effects while heterozygous *CCR5Δ32* provides a delayed progression of the disease after HIV infections (Liu, et al. 1996), (Huang, et al. 1996). As HIV need CCR5 protein on CD4 cells' surface so that they can adhere themselves to the surface and infect the cells but with homozygous *CCR5Δ32* mutation of the donor, the HIV can no longer enter the cells, granting a nearly complete immunity. An ex vivo treatment on hematopoietic stem cells were conducted in which genes of the aforementioned natural resistance to HIV were introduced to this cells. The hematopoietic blood cells were isolated from Brown's bone marrow cells as these cells are able to differentiate to all types of blood cells (Yucel and Kocabas 2017). These hematopoietic stem cells were then introduced back into Brown's body and as those stem cells replicated and differentiated into other blood cells his body was starting to be filled with all sorts of blood cells immune to AIDS. Brown is both HIV-1 and ARTs free since his clinical trial (Allers, et al. 2011), (Hütter, et al. 2009).

Brown remained anonymous and kept his name as the Berlin Patient until late 2010 when he decided to support research for curing HIV and to not be the only person who was cured of HIV (Lederman and Pike 2017). Along with some failed cases (Hütter 2014), treatment of HIV with Brown has been replicated with only a second patient by now so some scientists regard to this breakthrough as Armstrong's first step on the moon. The genotyping of *CCR5Δ32* is the critical part of the donor as other HSPC transplantations with wild type *CCR5* failed to be curative (Henrich, et al. 2013). It can be done but a lot more work has to be done to achieve this treatment on a large producible scale (Lederman, et al. 2016) also Brown had gone through a lot of conditioning as a part of his treatment and also he experienced graft-versus-host disease (Cannon, et al. 2014). It is a lot more likely to claim that a functional cure will be attained for treatments instead of obtaining a complete eradication of HIV in humans as that would be immensely harder than a functional cure. The HIV is likely to persist in some parts of the body (Fletcher, et al. 2014) and just as it did with Brown, a graft-versus-host disease can occur making it more dangerous (van Lunzen, et al. 2011), still a functional and large scalable cure is very desirable but it will require rigorous and numerous pre-clinical safety experiments (Corrigan-Curay, et al. 2015).

After Brown's clinical case a lot of gene editing studies has started aiming to obtain permanent and complete CCR5 absence without any harmful effects regarding incomplete or non-permanent proteins (Cannon and June 2011) along with multiple treatment strategies based around RNAs are also in development (Anderson and Akkina 2005), (Yang, et al. 1997), (Qin, et al. 2003).

1.3. *CCR5* disruption of CD4$^+$T cells via ZFN

ZFN was the first used genome editing method for evaluating CCR5-negative cells in clinical trials based on T-cell adoptive transfer experiences conducted before. Open reading frame (ORF) of *CCR5* was DSB cut at about 160th nucleotide, via an identified ZFN pair (Perez, et al. 2008). In pre-clinical studies where primary CD4$^+$ T cells were used, the anti-HIV efficiency of these ZFNs were first demonstrated. Most frequent result of these ZFNs is an addition of 5 nucleotides which is about 25% of all modified alleles. Of all these gene modifications, the most common effect was a premature stop codon introduced by this 5 bp duplication (Holt, et al. 2010). This genetic modification also makes it possible to determine an estimation of the overall frequency of edited *CCR5* genes since the introduced in-frame stop codons can also act as genetic markers. About 40 to 60% of all *CCR5* alleles can be disrupted by these ZFNs which are introduced by a chimeric Ad5/F35 adenovirus (AV) vector. Additionally in 33% of these modified cells both of the alleles were turned *CCR5* null by this gene disruption.

Modified *CCR5* alleles threefold proportion was observed to be increasing by the HIV-1 challenge in a model of mouse xenotransplantation which confirms the gene edited cells' expected survival advantage. After their HIV-1 challenge, mice had less viremia and were able to preserve their CD4$^+$ T human cells after they received *CCR5* ZFNs edited cells. Afterwards, ZFNs delivery of Ad5/F35 ro CD4+ T cells that were CD2/CD28-stimulated was also scaled to clinical use which paved the phase 1 clinical studies' way and made it possible to produce more than 10^{10} *CCR5* edited cells (Maier, et al. 2013).

The copy numbers, locations and the added transgene's profile of expressions will be maintained with far better control by anti-HIV genes that are inserted via HDR-mediation, when compared to standard approaches involving lentiviral vectors. Aside from the anti-HIV genes, gene editing will additionally allow HIV-specific chimeric antigen receptors to be precisely inserted into T cells and immunotherapy's latest advancements will be used to destroy cells that are infected with HIV (Sather, et al. 2015).

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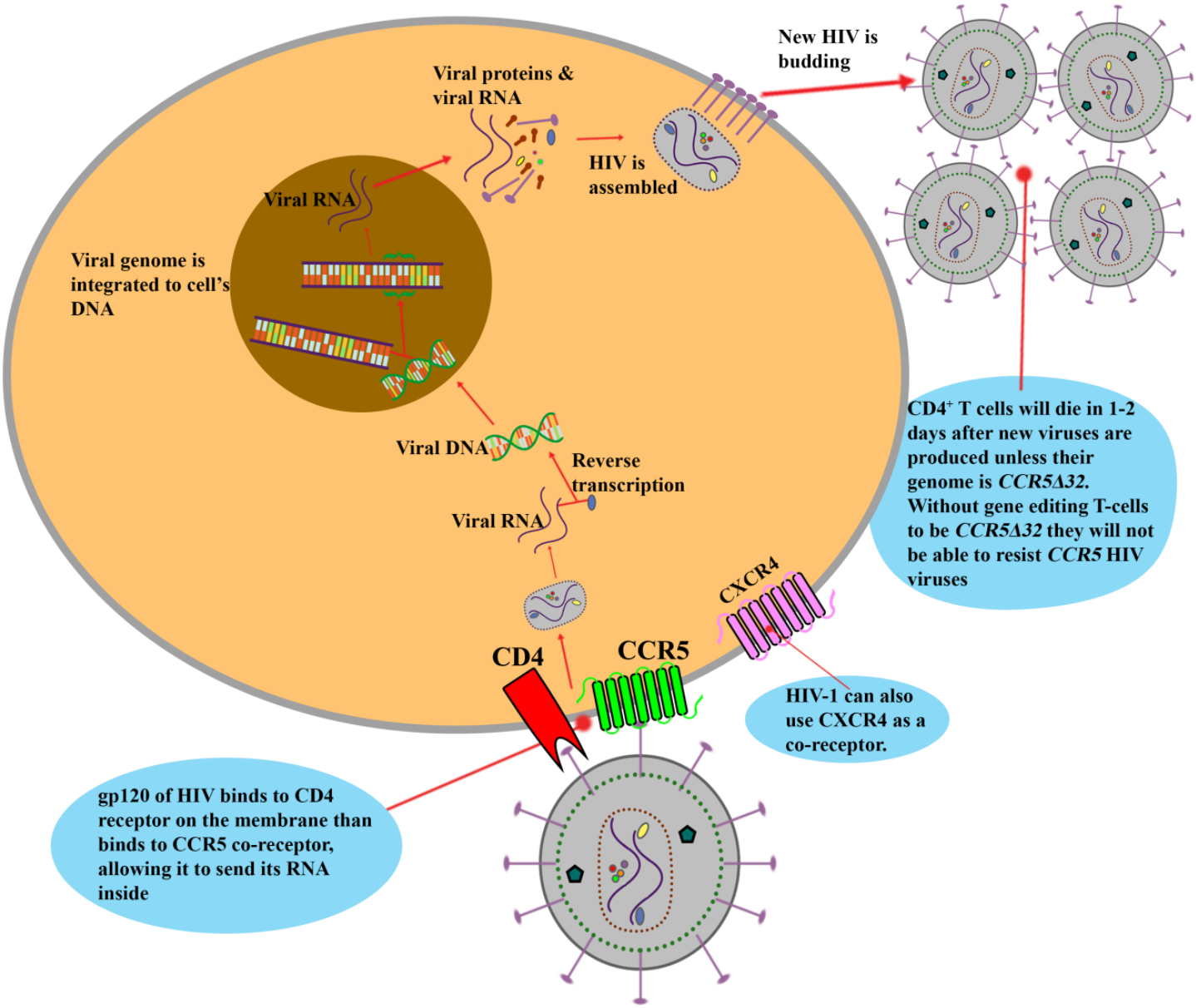


Figure 3. Two coreceptors are possible for HIV to use in order to deliver its genome. Before producing new HIV, the viral genome can remain latent for a long time until it is stimulated.

1.4. CRISPR-Cas9 mediated systems for HIV

Although the defense mechanism (Mojica, et al. 2009) of bacteria was first observed back in 1987 (Ishino, et al. 1987), its possible uses in experiments for scientists has been demonstrated only recently (Jinek, et al. 2012) which created a breakthrough for gene editing. Human CD34⁺ HSPCs were gene edited (De Ravin, et al. 2017) by the immensely improving and advancing CRISPR-Cas9 variations and techniques as its progression never even slowed down and more treatment strategies kept appearing (Niethammer, et al. 2018). The Cas9 part of the system recognizes and cuts the sequence of DNA/RNA a few base sequences away from the recognized protospacer adjacent motif (PAM) sequence (Hsu, et al. 2014). The Cas9 protein can be changed depending on the desired tests as different Cas proteins cut and recognize differently, like Cas12 which only cuts DNA or Cas13 which only cuts RNA (Cox, et al. 2017). Dead Cas9 (dCas9) for example can bind but cannot cut and as a result just sits at the site it bound, acting like an inhibitor (Konermann, et al. 2015). Another protein called Cpf1 can also be used where no tracrRNA will be required (Zetsche, et al. 2015) and with plentiful other options CRISPR, is a powerful, cheap and efficient gene editing tool that can multiplex and rivals use of ZFNs.

In the past few years CRISPR technology had been used especially with HIV-1 strains thanks to its very limited off target potentials and simplicity (Duan, et al. 2014). Ebina et al. (2013), have delivered via transfection CRISPR and *Streptococcus pyogenes* Cas9 (SpCas9) in 2013 while working with 293T, HeLa, Jurkat cells and they managed to suppress HIV-1 genes' expression successfully when targeting HIV-1 LTR in Jurkat cells

(Ebina, et al. 2013). TAR sequences of R region and the NF-κB binding cassettes which were at LTR's U3 region were the targeted sites. This study also managed to prove that integrated internal viral genes inside the infected host cells' genome could be exterminated with CRISPR methods therefore proving use of CRISPR-Cas9 variations to have high potential in treatment of HIV/AIDS. In 2014 Hu et al., also delivered CRISPR-SpCas9 via transfection while working with CHME5, TZM-BI, U937 cells and they also targeted same LTR-U3 regions in HIV-1 and succeeded in deactivating expression of viral gene with no noticeable off-targeting and very little genotoxicity. They managed to restrict replication of virus in a microglial cell line, a pro-monocytic cell line and in a T cell line which was infected latently (Hu, et al. 2014). In 2015, Liao et al., delivered CRISPR-SpCas9 via lentiviral vectors while the targeted region this time was the R region in LTR and the cell lines worked with were 293T-CD4-CCR5, 293T, hPSC (Liao, et al. 2015). Additionally, efficacy of excision and non-integrated pro-viral genome's disruption was shown to be increased by multiple targeting of HIV-1 genome's sites. In 2018, cutting latent HIV-1 provirus while also suppressing reactivation of it was shown to be possible by using a lentiviral vector which contained all the required components along with *Staphylococcus aureus* Cas9 (SaCas9) by Wang Q. et al., while they also demonstrated that, instead of using single sgRNA (single guide RNA) mediated SaCas9 editing, combining SaCas9/gRNAs provided higher efficacy at disrupting genome of HIV-1 with TZM-BI, C11 cells (Wang, et al. 2018).

Aside from using CRISPR-Cas9 technology and its variants to target HIV-1 genome, gene editing to block HIV-1's entry into the cells can also be accomplished (Cocchi, et al. 1995). As mentioned HIV-1 strain enters the cells after it has bound to receptor of CD4 and a co-receptor like CCR5 or CXCR4. CD4 however, is a key part of the functional human system so disrupting it is not a good possible strategy so instead, the aforementioned *CCR5* disruptions that were achieved via ZFNs to introduce homozygous *CCR5Δ32* mutation on their CD4 cells is aimed with CRIPSR-Cas9 methods (Xiao, et al. 2019).

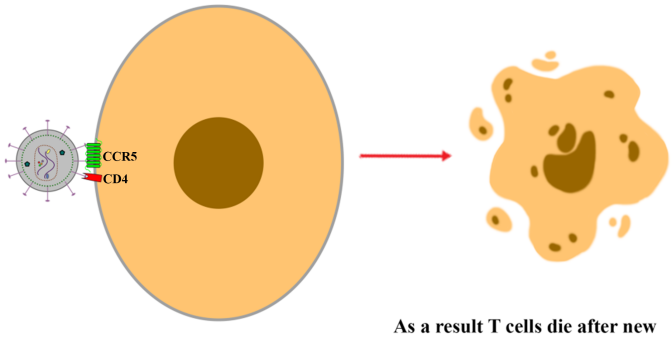


Figure 4. It takes about 24 – 48 hours for T cells to die after new HIVs emerge from inside of it.

Via transfection of sgRNAs and Cas9, human embryonic kidney (HEK) 293T cells' *CCR5* genes were silenced with SpCas9 by Cho et al. (2013), proving CRISPR to be useful but only at 13% efficiency (Cho, et al. 2013). Using the same delivery and target, Ye et al. introduced the *CCR5Δ32* mutation to induced pluripotent stem cells (iPSC) where he used piggyBac technology and obtained results that had an efficiency ranging from 33 to 100% (Ye, et al. 2014). When the iPSCs, that were genetically modified to have *CCR5Δ32* mutation, differentiated into macrophages, monocytes or other cells, they were resistant to infections from HIV-1. *CCR5* genes of $CD34^+$ HSPCs from K562 cell line were targeted with CRISPR-SpCas9 by Xu et al., in 2017 and HIV-1 infections were inhibited in vivo cells. In the secondary repopulation of the hematopoietic stem cells (HSC) the silenced expression of *CCR5* was still stable which provided a basis for the possible HIV-1/AIDS cures in clinical uses via transplanted *CCR5*-modified HSCs (Xu, et al. 2017).

The remaining host cells must be killed by using activation of antiviral immune responses and ARTs after the dormant HIV virus in the host cells are reactivated to achieve a complete eradication of the latent HIV reservoirs. This treatment strategy for HIV/AIDS is called as "shock and kill" (Kim, et al. 2018). Viral gene expressions have been shown to be reactivated by use of various drugs like the histone deacetylase (HDAC) inhibitor (Walker-Sperling, et al. 2016) which enhances expression of HIV-1 RNA in the latent reservoirs by remodeling and acetylation of chromatins (Archin, et al. 2012). Neither cell death nor destruction of the virus is achieved by the drugs as they only manage to induce latent HIV-1 in cells to be transcripted (Kim, et al. 2018). Cleaning the latent HIV-1 reservoirs with a higher efficiency can be achieved by using latent reversing agents (LRA) in combination and also the side effects of ARTs observed in patients might also be decreased (Yoder, et al. 2018). Targeting all of the latent viral reservoirs in this manner however, might not be possible and therefore not efficient enough as thought in theory (Rasmussen, et al. 2014). When the significant side effects that occur while patients use ARTs or/and HDAC inhibitors, are taken into account, producing new strategies for reactivating latent HIV-1 reservoirs is required.

Re-activation of latent HIV-1 viral reservoirs might be possible via CRISPR-Cas9 technology. dCas9 fusion proteins were used by various researchers in combination with sgRNAs which are specific to DNA target sequence's effector domains, in order to repress or activate gene transcription (Gilbert, et al. 2013), (Konermann, et al. 2015). The "shock and kill" strategy might actually be improved if transcription activator domains are fused with dCas9 which is catalytically inactive, as viral gene expression in HIV-1 reservoirs can be re-activated (Zhang, et al. 2015), (Kim, et al. 2017). Twenty-three sgRNAs were designed by Saayman et al., in order to target HIV-1 provirus' LTR U3 region, which resulted in them finding robust activation sites near binding sequences of NF-κB. The developed activation system managed to be more efficient when compared to latency reserving compounds like SAHA (Saayman, et al. 2016). Seven sgRNAs for targeting the functional key elements of HIV-1 LTR which includes NF-κB, U3 region, U5 region, R domain and Sp-1 binding sites, were designed by Limsirichai et al. Gene activation from HIV-1 LTR promoter could be induced with all of the designed sgRNAs while highly stimulating latent gene expression of HIV-1 was possible with 2 of the designed sgRNAs which overlapped with the binding sites of NF-κB and transactivation response elements (Limsirichai, et al. 2016). If CRISPR activators

were to be combined with SAHA, prostrain or other latency breaking reagents the re-activation process of HIV-1 latent reservoirs could be increased. CRISPR/Cas9-mediated systems therefore can be good alternatives or supportive agents in re-activating viral gene expression of HIV-1 latent reservoirs.

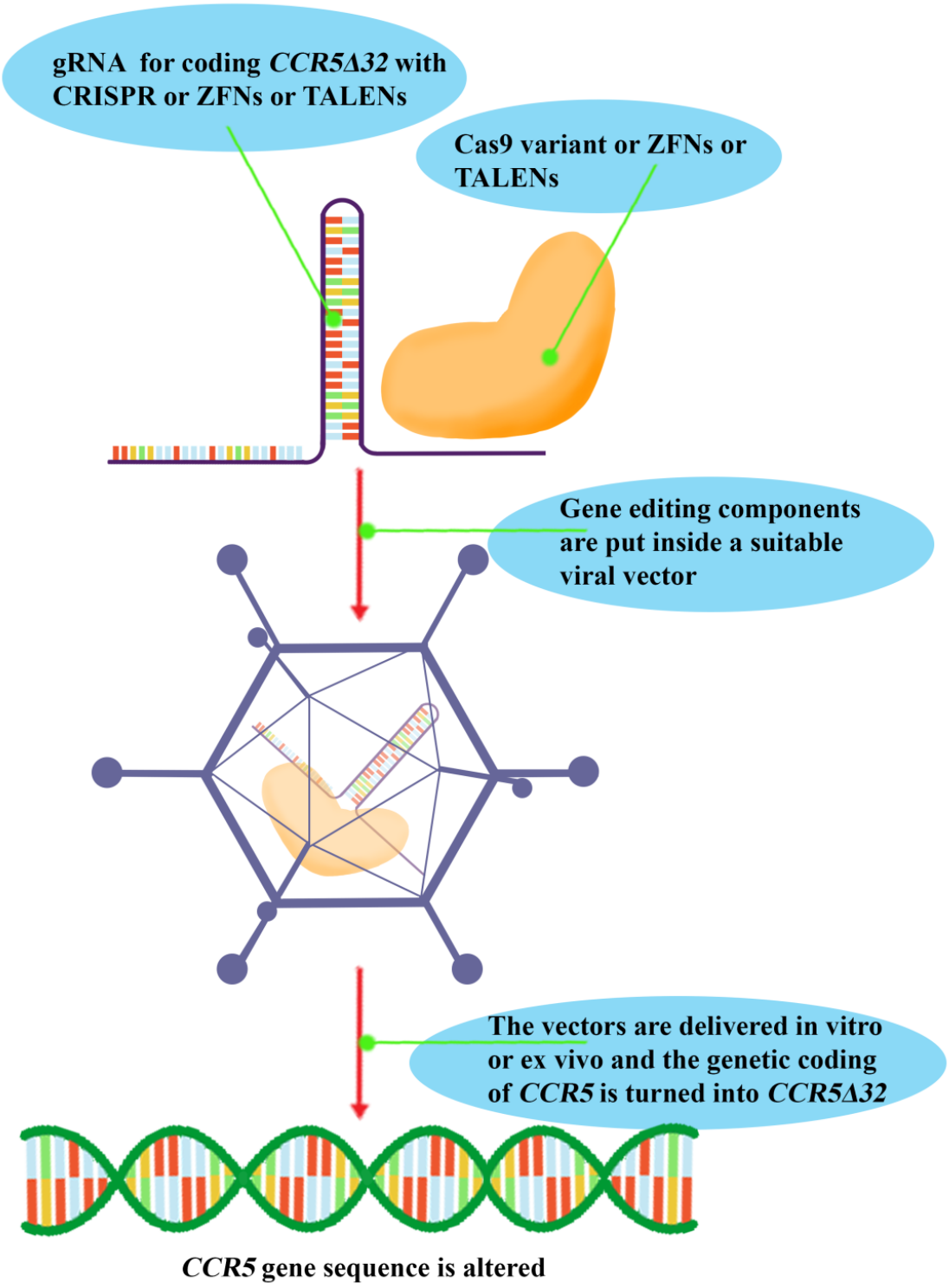
When HIV-1 or other viruses cause an infection various proteins in mammalian cells acts as restriction factors although these host factors are generally expressed weakly at infected cells (Chemudupati, et al. 2019). Expressing these restriction factors simultaneously might be an alternative strategy for preventing replication of HIV-1. In human cells the restriction factors APOBFC3B (A3B) and APOBEC3G (A3G) were tried to be induced via a Cas9 based approach by Borgerd et al., where they found out that usage of two sgRNAs is more efficient than usage of a single sgRNA and by inducing dC residues to dU residues (dC to Du) HIV-1 genome's editing, infection of Vif-deficient HIV-1 could be blocked by both of the activated proteins (Bogerd, et al. 2015). Unfortunately, regarding activating cellular host factors for inhibiting HIV-1 infections via usage of CRISPR/Cas9 technology, there are very limited studies. Some of the restriction factors that were discovered recently could be tested for their capability at being used in this application such as; serine incorporator five (SERINC5) which inhibits infection of viruses by preventing fusion of cells and viruses (Gonzalez-Enriquez, et al. 2017), human silencing hub (HUSH) or NONO which has been identified as a capsid-binding factor for Cyclic GMP-AMO synthase (cGAS)-mediated immune activation in dendritic cells and macrophages (Lahaye, et al. 2018). The incorporation of SERINC5 is prevented in virions that are newly generated by the HIV-1 accessory protein Nef, counteracting its function by redirecting it to a Rab7-positive endosomal compartment (Rosa, et al. 2015). Periphilin, MPP8 and TASOR make up the HUSH complex which can be degraded via DCAF1 dependent proteasomal pathway in primary T cells and HIV-2 infected cells, by the Vpx viral protein. In order to counter-react provirus transcription by HUSH-induced repression Vpx and Vpr from SIV, HIV-1 and HIV-2 can degrade the HUSH complex (Yurkovetskiy, et al. 2018). The conducted studies therefore suggest that HUSH complex is a critical host factor for HIV infections. CRISPR/Cas9 mediated methods can be simultaneously used to activate the expression of these mentioned representative restriction factors in infected cells. This will allow targeting various viruses at their different life phases since they can inhibit infection of HIV by several mechanisms.

1.5. Where CRISPR-Cas9 falls short

Although CRISPR mediated treatment systems are widely used in both mentioned fatal diseases that are caused by SNPs or in other dangerous and deadly diseases like Parkinson's disease (PD) (Zhou, et al. 2018), Duchenne muscular dystrophy (DMD) (Lim, et al. 2018) or various cardiovascular diseases, they do have their downsides and limitations that require additional work and research to overcome them.

CRISPR-Cas9 systems has off-targeting potential and inserting the genes between incorrect sequences can lead to very harmful mutations and chromosomal translocations like inducing leukemia (Kimberland, et al. 2018). Before the CRISPR systems were prevalent, gene therapies for gene editing even had a higher off target potential and still even with CRISPR mediated systems, reducing the off target effects is of high priority as the patient, who is already in a very weak and worn state will probably not be able to endure those off targeting side effects which were mostly the cases in the gene therapy trials in the past. When compared to TALENs or ZFNs however, the cutting of Cas9 is more precise and has a lot less off target chance. Truncated guide RNAs (Fu, et al. 2014) (tru-gRNA), dimerization dependent RNA-guided FokI-dCas9 (Wyvekens, et al. 2015) (RFN) or paired Cas9 nickases (Shen, et al. 2014) are results of various experiments trying to reduce off target chances. Gene editing with high efficiency in human HSPCs with decreased off target chances had been achieved in a study where a Cas9/gRNA ribonucleoprotein (RNP) complex with R691A SpCas9 mutant was used recently (Vakulskas, et al. 2018). In some cell types however, RNP application can trigger innate immune response and lead to cytotoxicity, limiting the use of this method. Immune response is caused by various factors like the administration route, the targeted tissue or the dose of applied CRISPR-Cas9 mediated system and the immunogenicity of such systems should be studied for the future clinical trial uses (Crudele and Chamberlain 2018).

As it was always an important factor to evaluate the delivering vectors for gene editing tools into human body for clinical trials, for CRISPR-Cas9 mediated systems this issue is still present. Due to their high efficiency viral vectors are more often used compared to non-viral vectors like liposomes. Mainly used viral vectors for CRISPR mediated gene editing systems were AV (Li, et al. 2015), adeno-associated viruses (AAV), and lentiviruses (Wang, et al. 2014). Herpes simplex viruses (HSV) were used in general gene therapy for targeting neural cells. Lentiviral vectors are used very commonly thanks to their high efficiency of delivery and lower risk of off targeting (Khalili, et al. 2017) since lentiviruses integrate into the host genome of both dividing and non-dividing cells with ease and mediate a stable expression (Wang, et al. 2014). Since the caused immunogenic effects of AV in clinical trials have been improved and they can carry larger DNA segments due to their rather high capacity, they are being used in numerous CRISPR-Cas9 mediated systems (SM Wold and Toth 2013) although recombinant AV generation might still represent significant limits (Afkhami, et al. 2016). AAV are also used due to their low toxicity, efficient delivery and being rather safe (Mingozzi and High 2013) but unfortunately their carriage capacity is low which limits the possible sequences that can be carried and due to their small packaging size re-application of them to deliver the whole sequence might be required which can induce immune responses due to multiple sessions (Zaiss and Muruve 2008). Both humoral and cellular immune responses had been found to be triggered by AAV in an early report however AAV are still considered rather an advantageous vector for gene therapy (Mingozzi, et al. 2009). If new AAV for CRISPR mediated systems are to be developed it must be taken into consideration to avoid chemical modifications that can create immune responses and immunosuppression and immunological



profiles should also be taken into account (Louis Jeune, et al. 2013).

Figure 5. Gene editing methods that are highly efficient like CRISPR or ZFNs are delivered via viral vectors and by interrupting *CCR5*, the CCR5 co-receptors become unavailable to HIV virus.

CRISPR-Cas9 mediated systems that are delivered via non-viral vectors have also been developed including liposome like lipid based reagents (Cardarelli, et al. 2016), polymer polyethyleneimine (PEI) (Li, et al. 2015) and nanoparticle based (Givens, et al. 2018) systems. However, since non-viral methods have lower efficiency, studies on improving the efficiency of the delivery have been done such as in a study where mouse models were being studied. A repair template to target and correct the gene hereditary tyrosinemia gene, fumarylacetoacetate hydrolase (FAH) and lipid nanoparticle captured mRNA of Cas9 with AAV encoding a sgRNA were tried to be combined in the mentioned study (Yin, et al. 2016).

When it comes to delivering CRISPR mediated systems into the brain for eradicating the latent HIV reservoirs in the CNS of infected patients, the biggest issue is the blood brain barrier (BBB). Large molecules are blocked and their transportation is not possible while only a certain amount of lipophilic group of molecules are allowed inside by BBB. There are some various strategies developed for overcoming BBB's difficulties such as intracerebroventricular infusion (ICV injection) or intracerebral injection strategies but they are rarely considered since they have a risk of causing brain damage. Nanoparticle based delivery of drugs however, can be an alternative strategy to overcome difficulties of BBB. Magnetic nanoparticles (Nair, et al. 2013), polymer nanoparticles (Fornaguera, et al. 2015) and gold nanoparticles (Mout, et al. 2017) are some of the various successfully used nanoparticles for brain target receptor-mediated transcytosis. For generating less cytotoxic side effects many novel nanoparticle investigations are being done for carrying CRISPR-Cas9 mediated systems into cells. When formulated differently, nanoparticles might have predilections for different tissues and organs such as liposome based ones being more preferable for lungs while different particles will be more suited for liver (Givens, et al. 2018).

Although CRISPR-Cas9 mediated antiviral tools are possible, HIV-1 has its own escape mechanism evolved around it. In a study Cas9/gRNA was found to inhibiting HIV-1 replication from which HIV-1 managed to escape later due to induced mutations around the cleavage sites that were induced by NHEJ repair mechanisms (Wang, et al. 2016). It was also demonstrated by other studies that CRISPR-Cas9 mediated systems can cause mutated viruses which can resist Cas9-sgRNA via generating DNA repair in host cells (Yoder and Bundschuh 2016). For dealing with mutant virus' escape mechanisms solutions like modified sgRNAs, suppressing the NHEJ activity and reprogrammed Cas9 nuclease had been suggested as possible solutions (Liang, et al. 2016). CRISPR-Cas9 related negative findings like these cause the scientists to be careful when designing sgRNAs and applying CRISPR related techniques for HIV/AIDS treatments in future clinical trials.

2. Conclusions

Development of gene editing applications are finding even more use with the unique and deadly disease of HIV/AIDS recent clinical trials of both Brown and "London Patient". Integrated HIV genomes can be targeted directly, or *CXCR4* and alternative HIV-1 co-receptor can be disrupted by the nucleases that are specially engineered in the future however ARTs are still the main clinical treatment strategy for HIV/AIDS. Although broadly neutralized antibodies had obtained promising results (Liu, et al. 2019) they still have to be tested for clinical applications rather than laboratory testing only.

Even though TALENs have lower off target chances than ZFNs and CRISPR, and are more flexible when it comes to DNA target designing, their high cost and time consuming production decreases their preferability. CRISPR however, is highly advantageous, with less off target effects, easier and cheaper construction, flexibility and multiplexibility so it is a highly preferable clinical tool overall. ZFNs did also achieve promising results in clinical trials as mentioned.

The more details we learn about the life cycle of HIV, latent viral reservoirs and other responsive cellular mechanisms, the more closer we get to complete HIV eradication by gene editing via technologies such as TALENs ZFNs or CRISPR.

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Gene editing studies for the treatment of anemia

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Abstract: With the uprising advancements in the genome editing technologies, it is now possible to modify and edit targeted DNA sequences with programmable endonucleases. The genome editing technologies have become more widely used by researchers after the discovery of zinc finger nucleases (ZFNs) and the transcription activator-like effector nucleases (TALENs) followed by the development of another revolutionary gene editing tool CRISPR-Cas9 system. Improvements in these promising gene editing tools not only reform researchers' understanding of the human genome but also serve as potential therapeutic approach for inherited blood disorders. The patients who have been suffering from inherited blood disorders are in need of novel therapies as available treatments are limited. Here, in this review, promising new gene editing technologies for the treatment of hemoglobinopathies including β-thalassemia and sickle cell disease are discussed.

Key words: HSC, CRISPR/Cas9, gene editing, Anemia, SCD, Thalassemia

1. Introduction

The novel approach called genome editing has been widely used in the research field of gene therapy, functional genomics and development of transgenic organisms for the past years. Gene editing is simply based on the usage of engineered, programmable and target specific nucleases inducing point specific modifications in the genome. These programmable nucleases are composed of a motive or sequence specific DNA binding domain and a DNA cleavage domain. DNA cleavage domain creates a double strand break (DSB) and facilitates the generation of insertions, deletions and substitutions desired at the genomic site of interest. Various platforms of engineered nucleases have been in use for genome editing studies. One of the most widely used and pioneer gene editing system is Zinc Finger Nucleases (ZFNs) (Bhakta, et al. 2013; Cathomen and Keith Joung 2008; Kim, et al. 2011; Townsend, et al. 2009). ZFNs consist of a global Cys_2-His_2 DNA binding domain and a DNA cleavage domain named FokI endonuclease (Kim, et al. 1996). Another popular tool of genome editing technology is transcription activator-like effector nucleases (TALENs), a protein originated from pathogenic bacteria named Xanthomonas (Cermak, et al. 2011; Reyon, et al. 2012; Wood, et al. 2011). TALENs provide specific nucleotide recognition by their DNA binding domain composed of amino acid motives. Each of these conserved motives, which are robustly programmable in a target specific manner, recognize a particular nucleotide (Briggs, et al. 2012). The most recently, clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR-associated protein (Cas) 9 was introduced to dethrone TALENs and ZFNs for gene editing (Cong, et al. 2013; S. Makarova, et al. 2011). Unlike a peptide – DNA interaction to provide targeting specificity as in ZFN and TALEN approach, CRISPR/Cas9 system has a basis of guide RNA (gRNA) – DNA complementation to ensure a higher performance of sequence specific targeting of any genomic location. A 20 base long guide RNA sequence co-delivered with Cas9 protein is the only requirement for specific targeting and DNA cleavage (Jinek, et al. 2012). CRISPR technology promises a faster, easier and cheaper design compare to ZFN, TALEN and related genome editing techniques. Comparing the efficiencies of CRISPR/Cas9 and TALEN systems applied on the same cell line, it was observed that CRISPR/Cas9 is more robust, and promising method for effective genome editing (Ding, et al. 2013).

CRISPR/Cas9 system was first discovered as an acquired immunity machinery in bacteria. Invader DNA is recognized by CRISPR RNA (crRNA) and cleaved by Cas9 nuclease (Gasiunas, et al. 2012). In bacterial and archaeal genomes CRISPR locus is made of strictly conserved repetitive DNA sequences interspaced with specific sequences called spacers. Spacer sequences are generated through cleavage of invader's DNA into small fragments and integration into CRISPR locus of the host genome. These spacer sequences are then used as DNA templates to produce crRNA targeting viral or phage DNA, acting as bacterial immunity library members. There are different CRISPR/Cas9 systems, which have been still identified and also engineered, based on amino acid sequences and tertiary structures of Cas9 protein. Major classes of CRISPR/Cas9 system are I, II and III. It was described that class II CRISPR/Cas9 system requires only a Cas9 protein with two nuclease domains named RuvC and HNH, incorporated with a guide RNA. Thus, class II CRISPR/Cas9 has been pointed as a relatively simpler, efficient and easily designable system for gene editing studies (S. Makarova, et al. 2011).

CRISPR/Cas9 gene editing system is based on generation of a DSB followed by the process of cellular DNA repair. The original CRISPR/Cas9 system is guided to target site by the combination of mature crRNA and trans activating crRNA (tracrRNA) which is partially complementary to crRNA and provides to maturation

of crRNA (Zhang, et al. 2014). In research applications, a chimeric RNA, containing both crRNA and tracrRNA sequences, called as guide RNA is used (Jinek, et al. 2012). Guide RNAs varying between 20-24 nucleotides are able to be designed using variety of tools, providing easy application ability. Target specific cleavage of DNA also requires another component called protospacer adjacent motif (PAM) which is a 2-6 base length DNA sequence located in the downstream of target site (Shah, et al. 2013). PAM sequence is essential for successful binding and cleavage of targeted genomic loci(Jinek, et al. 2012; Mojica, et al. 2009; Sternberg, et al. 2014). The most commonly used PAM sequence is 5'-NGG-3' associated with Cas9 nuclease of Streptococcus Pyogenes and the researchers are still studying to identify different PAM sequences to achieve improved targeting in a wider range of sites on genome (Anders, et al. 2014; Esvelt, et al. 2013). Together with gRNA and PAM sequences, CRISPR/Cas9 system can target up to 30 base-length on target site, which is theoretically a unique sequence on whole genome of different organisms. However, it has been reported that CRISPR/Cas9 system has a tolerance to mismatches observed between the guide and target sequences, which would lead to off-target mutagenesis (Cong, et al. 2013; Fu, et al. 2013; Mali, et al. 2013).

The fundamental logic behind genome modification using engineered nucleases is the generation DSB near target site, triggering a subsequent DNA repair process (Gasiunas, et al. 2012; Kim, et al. 1996; Wood, et al. 2011). There are two main endogenous repair mechanisms for DSB, which are non-homologous end joining (NHEJ) and homology directed repair (HDR). In NHEJ, broken ends of DNA are directly ligated back together in brief. In most of the cases, NHEJ repair mechanism results in small insertions or deletions (in-dels) at the site of DNA break. These in-dels would result in small sized mutations causing gene silencing. The second repair mechanism to get rid of DSB is HDR. In this mechanism, a homology containing sequence of DNA serving as a template is required to synthesize new DNA repair the break by homologous recombination. Naturally, a sister chromatid is the template for HDR in case of DSB repair. However, it is reported that HDR mechanism can also work with the presence of an externally introduced DNA bearing homology regions, called as donor template (Gratz, et al. 2013; Zhang, et al. 2014).

Genome editing has shown to have a remarkable potential to cure genetic diseases through permanent correction of mutations (Sebastiano, et al. 2011; Urnov, et al. 2005; Zou, et al. 2011) or insertion of recuperative DNA sequences as done in gene therapy (Torikai, et al. 2012; Voit, et al. 2013). Gene editing technology enables targeted genome modifications with higher precision and adaptability.

1.1. Gene editing of blood cells

Gene editing of blood cells as therapeutic approach to cure blood disorders is a simple and conceptual idea, which has been intensively focused for the last several years in the field of research. Initial scientific approach for genome editing of blood cells had been based on use of viral vectors such as retrovirus and lentivirus derived vectors. Integration of these vectors into host genome is operated in an uncontrolled manner, resulting in unexpected side effects. Several studies have underlined that lentiviral and retroviral vectors do not demonstrate random genomic integration but biased integrative fashion(Bushman, et al. 2005). Various concerns have emerged due to lentiviral vector usage in treatments of blood disorders such as leukaemia and lymphoma caused by proto-oncogene activation upon insertion of viral genomic content. However, it has not been reported that there is a high risk of leukaemia in humans and lymphoma formation in mice, remaining a risk of long-term latency.

Gene editing technology is currently a substitutive method to gene therapy applications conducted by usage of genome integrative viral vectors to achieve permanent genetic modifications on target genes. Gene editing technology proposes modifying a genome with high control, fidelity and it claimed as a promising approach for treatment of hereditary blood disorders. Using the tools such ZFN, TALEN and CRISPR/Cas9, it has been reported that various haematological conditions caused by genetic background are able to be ablated (Meissner, et al. 2014; Porteus 2015) In this paper, the possible applications of gene editing technologies for treatment of different blood disorders caused by mutated genes is reviewed.

1.2. Gene editing in hematopoietic stem cells

Hematopoietic stem and progenitor cells (HSPCs), located in bone marrow, are multipotent cells which are the main resources of mature blood cell generation through haematopoiesis (Doulatov, et al. 2012). HSPCs consist of 0.1% of the total cell population in bone marrow and capable of self-renewal and differentiation (Morrison, et al. 1995). Since HSPCs are easily isolated, characterized, manipulated and capable of reestablishment of a complete and functional hematopoietic system, bone marrow transplantation (BMT), also called hematopoietic stem cell transplantation (HSCT), has been proposed as a therapeutic application for blood and immune disorders (Thomas, et al. 1975). BMT can be applied in two ways; autologous or allogenic. In autologous HSCT, patient-derived cells are manipulated in vitro and re-engrafted. Allogeneic transplantation is based on engraftment of healthy donor-derived HSPCs for the treatment of inherited blood disorders. Nonetheless, allogeneic transplantation accompanies drawbacks and clinical complications such as graft versus host disease (GVHD) in which engrafted cells react against receiver's cells, leading severe immune attack based health issues (Stolfi, et al. 2016). Furthermore, availability of suitable donors is a major concern as in all types of tissue and cell transplantations. To overcome the hurdles of allogeneic transplantation, in vitro manipulation of autologous hematopoietic stem cells to generate healthy cells has been favoured as a promising therapeutic approach for inherited blood disorders.

The concept of gene therapy has emerged as a promising tool to cure inherited blood disorders. Viral delivery of desired genes or fragments using modified virus constructs demonstrated success to restore gene expression deficiencies (Kaufmann, et al. 2013). Gene therapy can be carried out by addition, substitution or alteration of the gene of interest. These genetic manipulations can be achieved by both in vivo and ex vivo followed by reinfusion of modified cells back to patient (Naldini 2015). Up to the present, practise of gene therapy on hematopoietic stem cells has been an option to cure hematopoietic disorders such as

hemoglobinopathies and immunodeficiencies (Cavazzana 2014). Due to convenience of isolation, culture and reinfusion, HSPCs have been used for ex vivo gene therapy (Ghosh, et al. 2015). However, the inefficacy in controlling the gene delivery and genomic integration dosage and site have led various safety concerns such as oncogenic results due to potential insertional mutagenesis or activation of protooncogenes (Bersenev and Levine 2012; Kaufmann, et al. 2013). Thus, further improvements are required to alleviate potential drawbacks of gene therapy applications on HSPCs.

Ex vivo correction of HSPCs through transgene addition is a promising therapeutic method to provide stable expression and alleviate the disease caused by the malfunctioning gene. Viral vectors originated from retroviruses and lentiviruses have widely been used to achieve this strategy since they provide high efficiency in transduction and gene expression. However, viral delivery has shown adverse effects due to genotoxicity and insertional mutagenesis despite successful disease recoveries. In a study targeting adenosine deaminase deficiency in hematopoietic stem cells was resulted in T cell leukaemia in treated patients (Touzot, et al. 2014). Viral insertions have potential to cause activation of surrounding genes due to the presence of long terminal repeats (LTR) found in both retro and lentiviruses to facilitate genomic integration of the insert sequence. A potential activation of a proto-oncogene as side effect of viral vector delivered gene insertions would have a risk to bring severe consequences to patients. Thus, this therapeutic approach is required to be improved to eliminate safety concerns. Development of self-inactivating lentivirus vectors was reported to mitigate genotoxicity due to their modified genome characteristics and preferences of genomic integration sites in host (Amendola, et al. 2005). In clinical trials using self-inactivating lentiviruses on beta thalassemia patients, significant efficiency and safety improvement was observed (Naldini 2015). Nevertheless, potential adverse effects in longer post-treatment period should be assessed carefully and safety concerns should be minimized to implement gene addition based cell correction as a universal therapy method for inherited blood disorders and immunodeficiencies.

Discovery of genome editing strategies has provided more precise mutation repair in comparison to gene therapy. Engineered nuclease systems are strikingly promising tools for inherited disease therapeutics. Instead of gene addition, precise repair of mutant genes has appeared to be a safer approach. Tremendous effort is currently made to adopt and optimize the gene editing systems into iPSCs and HSPCs, carrying onward to globally accessible clinical applications. Inducing controlled double strand breaks on target locus and activating homology directed repair mechanisms in cells, engineered nucleases provide much more precise and effective way of disease therapy. Depending on the therapeutic strategy, gene correction, knock-in and safe harbour integrations are applicable in nuclease-mediated gene editing technology. Although NHEJ mechanism is more frequent than HDR, it is currently known that cells tend to favour HDR mechanism during S and G2 phases of cell cycle (Ciccia and Elledge 2010). Therefore, hematopoietic cells may be simultaneously induced to shift proliferative state and gene correction by delivery of engineered nucleases and repair templates to achieve high efficiency gene editing.

As with the gene therapy, delivery of gene editing components to HSPCs is a major bottleneck. To avoid genotoxicity and off-target mutations, transient expression or controlled inhibition of nucleases is currently in demand. Delivery methods of genome editing tools can be categorized into two, as viral and non-viral delivery. Considering non-viral delivery approach, cells can be transfected with plasmid DNA containing and expressing gene editing components, in vitro transcribed mRNA to induce translation of nuclease in host cell, or direct delivery of purified nucleases with donor repair templates (Skipper and Mikkelsen 2015). Donor template delivery is also versatile that it could be introduced to target cells in the form of plasmid DNA, dsDNA or ssDNA linear oligo (Chen, et al. 2011; Orlando, et al. 2010). In non-viral delivery of gene editing tools, transfections are supported by driving forces, which are cationic polymers, lipids, calcium phosphate and electroporation. However, the non-viral transfection efficiency varies between cell types. Combination of chemical supplements and transfection methods would result in an increase in efficiency but also toxicity and stress related cell death. Delivery of in vitro transcribed mRNAs encoding engineered nucleases (ZFN, TALEN or Cas9+gRNA) appears to be more advantageous due to lower genotoxicity and transient nuclease activity (Skipper and Mikkelsen 2015). Direct delivery of engineered nucleases as purified proteins using electroporation has been applied as another alternative approach to achieve temporary and safe genome editing (Kim, et al. 2014). However, the size of nuclease would affect the passage through cell membrane. To overcome this challenge, recombinant proteins with smaller size would be more convenient. In a recent study, genome editing of hematopoietic stem cells have been efficiently performed using CRISPR/Cas9 system in the form of ribonucleoprotein which is purified Cas9 protein and target specific gRNA complex (Liang, et al. 2015).

Apart from non-viral delivery strategies of genome editing tools, viral based methods are alternatively used. Advances in genome engineering have brought in improvements in viral transfer tools and methods. Generation of non-integrating viral vector such as adenovirus vectors (AdVs), adeno-associated viral vectors (AAVs) and integrase deficient lentivirus vectors (IDLVs) enabled successful transfer of genes required for the expression of gene editing tool in host cells in both in vitro and in vivo (Skipper and Mikkelsen 2015). Each viral vector mentioned above possesses different characteristics in terms of transduction efficiency, packaging size and target cell type. AdVs are dsDNA viruses enabling a packaging capacity up to 37 kb which provides enough room for genes encoding nucleases and donor repair templates. As a proof of concept, CD34+ T cells had previously been genome modified using ZFNs packaged in AdVs to acquire HIV-1 resistance (Perez, et al. 2008). AAVs are currently the most widely used ssDNA viruses coinfected with a partner such as adenoviruses or herpes simplex viruses. AAVs are advantageous due to their low immunogenicity and low frequency of random integration into host genome. However, the packaging capacity is quite low, around 4.7 kb, which might be incompatible for large sized nucleases and donor DNA (Flotte 2000). For instance, spCas9 can barely fit into a typical AAV,

leaving no space for other elements required for gene editing. In comparison to Cas9 and TALENs, ZFNs are encoded by smaller sequence that can be packaged and delivered using AAVs (Chira, et al. 2015). IDLVs has been used to deliver ZFNs and donor templates (Lombardo, et al. 2008). Regarding to their competence in transducing non-dividing cells and integration deficiency, IDLVs are considered as advantageous tools for packaging (Naldini 2011). Nevertheless, HSPCs are hard to transduce since they require higher titers of viral particles which is challenging to obtain for IDLVs. Therefore, IDVL production protocols ought to be developed to eliminate this drawback present in case of hematopoietic cell editing.

1.3. Correcting beta-thalassemia

Beta-thalassemia is an autosomal recessive blood disorder with a high prevalence in Mediterranean, Middle East and South-Eastern Asia (Colah, et al. 2010). Beta-thalassemia is mainly caused by the reduced or absent expression of beta globin subunit of haemoglobin protein which is the main carrier of oxygen (Rund and Rachmilewitz 2005). According to its severity level, beta-thalassemia is divided into three major states, which are beta-thalassemia carrier, beta-thalassemia intermedia, and beta-thalassemia major. Patients heterozygous to beta-thalassemia mutations are specified as beta-thalassemia carrier and do not exhibit clinical symptoms of the disease. Thalassemia major is mostly observed in homozygous mutant patients and it is the most severe level of the disease, requiring frequent blood transfusions to alleviate severe anaemia suffered. Unlike thalassemia major and thalassemia carrier, thalassemia intermedia possesses a wider range of severity in terms of clinical symptoms due to genotypical heterogeneity (Cao and Galanello 2010). Studies showed that over 300 mutations spotted on HBB gene located in chromosome 11 (11p15.5) causing beta-thalassemia (Kountouris, et al. 2014). Mutations on this locus result in significant reduction or depletion of beta globin expression. Lack of beta globin would cause reduced production of mature haemoglobin composed of tetramer of two alpha and two beta globin subunits. Depository of free alpha globin is known to result in defects on erythropoiesis and early apoptotic tendency in erythroid lineage (Galanello and Origa 2010; Rivella 2009).

Patients carrying homozygous mutations on HBB gene have been reported to suffer from severe anaemia as the major symptom, and require receiving blood transfusions and supportive drug administration as supportive and relieving medical care (Oliveri 1999; Olivieri and Brittenham 2013). Iron chelation therapy has been one of the most popular application for thalassemia related severe anaemia (Poggiali, et al. 2012). Bone marrow transplantation (BMT) or also called allogenic hematopoietic stem and progenitor cell (HSPC) transplantation has been the only clinically approved method for Beta-Thalassemia treatment (Angelucci, et al. 2014; King and Shenoy 2014). Like all types of graft transplantations, finding a suitable donor is the major concern. Post-transplantation complications also bear clinical conditions to overcome, such as graft versus host disease (GVHD) in which the transplanted tissue or cells begin attacking receiver body compartments. Challenges concerning BMT have urged researchers to find out possible alternative approaches (Finotti and Gambari 2014; Gambari 2012).

Recent improvements have accelerated and expanded the research of personalised and genome based therapies to cure genetic diseases. The concept of gene therapy has been mooted to apply for genetic diseases as the beta-thalassemia. Delivery of viral vectors carrying wild type HBB gene to HSCs has been proposed an alternative method of BMT (Cavazzana-Calvo, et al. 2010). Lentiviral expression of exogenous beta globin was proposed as a novel approach for beta thalassemia treatment. Most of the efforts have been dedicated to achieving optimized viral transduction of HSPCs with high efficiency (Miccio, et al. 2011; Puthenveetil, et al. 2004; Roselli, et al. 2010). However, safety concerns have emerged due to potential random integration of these viral vectors, triggering mutations or activation of proto-oncogenes causing lymphomas and other genotoxic events (Cesana, et al. 2014; Nowrouzi, et al. 2013; Woods, et al. 2006). Therefore, alternative therapeutic approaches overcoming technical challenges and post-treatment complications have been under investigation. For the gene therapy applications, HSPCs are the main target cells. However, the access to HSPCs, expansion in vitro and yield of viral transduction are still challenging and needs to be improved through development of supportive methods (Wilber, et al. 2011). It is clearly observed that majority of the studies, aiming gene therapy or genome editing for the treatment of hemoglobinopathy disorders, use alternative substrates instead of HSPCs. More work is required to enhance the usage of patient derived HSPCs for clinical methods to cure blood disorders such as beta-thalassemia.

Development of induced pluripotent stem cells (iPSCs) technology broadens the horizons of patient specific and regenerative medicine (Csobonyeiova, et al. 2015; Kim 2014; Takahashi and Yamanaka 2006). Generation of iPSCs by induction of reprogramming factors (Sox2, Oct3/4, Klf4 and c-Myc) from somatic cells would enable production of healthy cells, provided by the correction of mutations causing disease. iPSCs technology accompanied with gene therapy or gene editing has been presented as a novel therapeutic approach due to its possible usage as patient specific material. It was shown that human iPSCs preserve HBB gene expression characteristics after in vitro erythroid differentiation, suggesting that these cells are promising materials for gene therapy to cure beta Thalassemia and sickle cell disease (Dias, et al. 2011; Kobari, et al. 2012). The basic concept relies on generation of genetic editing of iPSCs carrying mutation and autologous transplantation of subsequently differentiated healthy cells into patients.

Current approaches for the treatment of disorders led by primary mutation have gravitated to DNA level of repair. Instead of gene therapies carrying risks of insertional mutagenesis induced by viral vectors, direct genome editing has been suggested as a novel tool. In general, the major issue of the genome editing methods to cure inherited blood disorders has been the low yields of editing in HSPCs. Recently, a group of researchers have reported that they achieved a targeted genome editing in HSPCs obtained from patients suffering X linked severe combined immunodeficiency, with a success rate of 3-11% in different

subgroups (Genovese, et al. 2014). Efficiency of genome editing in patient derived HSPCs should be improved to be an approved clinical application in the future.

Several studies have discussed possible applications to correct mutations on HBB gene, resulting in beta-thalassemia. It was reported that iPSCs derived from beta-thalassemia patients was achieved to be corrected using TALEN method which is described as a robust and non-viral, non-integrative approach (Ma, et al. 2013). In this study, TALENs were designed to target 3' downstream of HBB gene to induce DSB, co-delivered with a donor template carrying wild type HBB sequence to correct the mutation through HDR mechanism. Gene edited iPSCs were remained as pluripotent with a normal karyotype and able to differentiate into HSPCs, followed by further differentiation to erythroblasts expressing wild type beta globin. Their results showed that TALEN method was an effective approach to correct different beta-thalassemia mutations observed in two different patients, supported by the repaired function of HBB gene in HSPCs differentiated from integration-free and patient specific iPSCs.

ZFNs and TALENs have been widely used for the specific and efficient alterations of endogenous genomic loci (Hockemeyer, et al. 2009; Katada and Komiyama 2011). Alongside TALEN and ZFN technology, CRISPR/Cas9 system has been investigated for targeted modification of beta-globin (Cradick, et al. 2013; Voit, et al. 2013). Recent years CRISPR/Cas9 technology has been the most popular and promising method to correct small sized mutations. Beta-thalassemia is one of the target blood disorder to study using CRISPR/Cas9 system. In a study, researchers have reported that they achieved to correct homozygous HBB point mutation in iPSCs generated from patients suffering from beta-thalassemia. It was demonstrated that one of the mutated alleles was succeeded to be corrected by CRISPR/Cas9, supported by the data showing edited cells with normal karyotype. It was underlined that iPSCs remained full pluripotency after gene editing. Additionally, $CD34^+$ / $CD31^+$ progenitor cells derived from corrected iPSCs demonstrated repaired expression of HBB and improved potential of hematopoietic differentiation (Song, et al. 2014). This study claims that mutation corrected patient specific iPSCs can be a promising method to treat beta-thalassemia using CRISPR/Cas9 technology.

1.4. Correcting the sickle cell disease

Sickle cell disease (SCD) is known as a monogenic blood disease caused by a point mutation in human β-globin gene (HBB) encoding two subunits of tetrameric protein haemoglobin. Malformation of the haemoglobin protein structure leads formation of abnormally shaped red blood cells. SCD is caused by mutant copies of HBB called haemoglobin S (HbS). The point mutation, substation of A to T, in the sixth codon of HBB gene results in conversion of glutamic acid to valine and consequently an abnormal folding of haemoglobin emerges (Frenette and Atweh 2007). Abnormal HbS haemoglobin results in aggregation and polymerization of the protein, forming sickle shaped red blood cells. Unlike doughnut shaped, elastic normal red blood cells, sickle cells have a stiff and sharp sticky structure that easily aggregate and stick on narrow blood vessel interior surface. As the outcome of occlusion, insufficient oxygen is delivered to tissues and therefore organ damages are observed in long term of disease progress (Ashley-Koch, et al. 2000). Moreover, sickle shaped red blood cells have remarkably shorter lifespan compare to normal red blood cells, causing chronic anaemia as a further pathological impact on patients health (Azar and Wong 2017). Clinically, the presence of homozygous variant (HbSS) is the most severe case for SCD patients, in comparison to heterozygous mutants (Frenette and Atweh 2007). It has been reported that around 100,000 patients have been diagnosed with SCD in USA (Hassell). Approximately 300,000 children is born with SCD worldwide each year (Piel 2016). Despite of high frequency of SCD, still there is not any definitive treatment for this disease. Current treatments are predominantly available as supportive agents to reduce disease severity and background complications. Mostly used clinical applications for SCD patients are blood transfusion, hydroxyurea therapy and vaccinations to prevent the risk of severe infections which SCD patients are prone (Aliyu, et al. 2006).

Regarding that mature blood cells are derived from hematopoietic stem and progenitor cells (HSPCs), a clinical approach called allogenic stem cell transplantation has been used as a promising treatment for SCD and similar blood disorders. This technique relies on finding suitable donors, harvesting healthy HSPCs and transplanting to the patients (Shenoy 2011). Despite the successful results of this method, unfortunately it is not a universal treatment due to lack of available donors, immune response effects such as graft versus host disease (GVHD) and other side effects with several toxicities (Locatelli and Pagliara 2012). To eliminate the drawbacks of allogenic transplantation, autologous transplantation of ex vivo corrected HSPCs has been proposed as a promising method. Monogenic blood diseases such as SCD could be cured by direct correction of mutations using genome editing tools which are also called engineered nucleases. These nucleases have demonstrated high potential for therapeutic applications in previous studies (Abil, et al. 2014).

Previously, ZFNs and TALENs had been reported to successful for targeting and correction of SCD mutation on HBB gene up to a certain extend. Patient derived iPSCs with SCD mutation have been targeted using ZFNs with 9.8% efficiency (Sebastiano, et al. 2011). A similar study have reported that iPSCs of SCD patients have been corrected without disturbing the subsequent differentiation efficiency into erythroid cell line however β-globin expression levels remained lower than healthy subjects (Zou, et al. 2011). Hoban et al. recently performed delivery of ZFNs in CD34+ HSPCs with up to 65% DSB induction rate. Despite the level of gene correction was 10-20%, repopulation of engrafted hematopoietic cells in bone marrow and spleen of immunocompromised mice remained insufficient for long term consideration (Hoban, et al. 2015). The therapeutic potential of TALENs has also been investigated for SCD. Engineered TALENs was introduced the cells to induce DSB around the SCD mutation in HBB gene to demonstrate efficient targeting (Sun, et al. 2012). A follow up study was published using iPSCs with promising results of targeting efficiency (Sun and Zhao 2014). Another group have showed that TALENs was a promising genome editing tool to correct SCD mutation in patient derived

iPSCs. Corrected iPSCs were further differentiated into erythroid cells and the results demonstrated that 30-40% of the cell population with heterozygous wild type phenotype, which is clinically sufficient (Ramalingam, et al. 2014). There has not been any study published concerning the SCD mutation correction in HSPCs using TALEN platform.

Recently, type II CRISPR/Cas9 system is the most fashionable tool for genome editing and promising approach for the direct correction of mutations causing monogenic diseases as SCD (Wright, et al. 2016). In comparison to ZFNs and TALENs, CRISPR/Cas9 system exhibited higher efficiency and lower cost while controlled targeting of HBB gene in iPSCs and K562 cell line which is originally derived from a patient with chronic myeloid leukemia (Cottle, et al. 2015; Huang, et al. 2015). Huang et al. successfully corrected the mutation causing SCD, applying CRISPR/Cas9 system in iPSCs of SCD patients. In this study, researchers accomplished to preserve differentiation ability of edited iPSCs into erythrocyte cells with improved levels of β-globin expression (Huang, et al. 2015). Liang et al. gave a new impulse to HBB gene editing technology applying CRISPR/Cas9 system in human zygotes. However, various off-target mutations induced by Cas9 activity and low efficiency of HDR were recorded, requiring more investigation for improvement (Liang, et al. 2015). In a recently published study, researchers attained a significant success at HDR efficiency through application of CRISPR/Cas9 system in CD34+ HSPCs (DeWitt, et al. 2016). DeWitt et al. introduced Cas9 and gRNAs as ribonucleoprotein complex along with ssDNA donor templates for desired correction. Researchers concluded that their study resulting in high gene editing rates for HBB locus and clinically significant recovery of WT β-globin production. Contrast to the previous attempts, lower but detectable off-targeting activity was observed in both HSPCs and K562 cells. Although the previous and recent scientific work has held promise for SCD patients, more research is required to alleviate off-targeting activity of CRISPR/Cas9 system and upgrade HDR yield in order to carry genome editing one step forward to clinical trials and become a universal therapeutic application.

2. Conclusions

Hemoglinopathies are a crucial international healthcare problem as being the most common monogenic diseases all around the world. The most clinically severe inherited blood disorders are the sickle cell diseases (SCD) and β–thalassemia, thus, they are suitable for treatment by genome editing technologies. Engineered nucleases like ZFN and TALEN, as well as CRISPR/Cas9 system broadened the area of regenerative medicine based treatments for hemoglinopathies and served a safer approach by precise repair of mutant genes rather than gene addition. Even though gene editing approaches are desired approaches for the treatment of hemoglinopathies, the delivery of gene editing components to HSPCs remain as a significant drawback. Transient expression or controlled inhibition of nucleases is recently favored so as to prevent genotoxicity and off-target mutations. Researchers are adopting and optimizing new gene editing systems into iPSCs and HSPCs to provide ease of access for the clinical treatments. Possible treatment options could be found in the near future with the elevated rate of new discoveries in the genome-editing field.

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Alternative Therapies to Antibiotics: CRISPR-Cas antimicrobials

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Antibiotics affect specific mechanisms of bacteria by targeting cellular pathways or functions such as function of cell membrane, blockage of cell wall synthesis, protein or nucleic acid synthesis. They can't selectively kill targeted pathogens in the mixed microbial population with these mechanisms. Antibiotics cause dysfunction not only of the bacteria that cause infection but also of the beneficial microbiota members in the host. Currently, there is no specific antibiotic strategy targeting only virulent or antibiotic-resistant bacteria. Current antibiotic strategies aren't specific; resistant bacteria allow spread of the resistance genes in the bacterial population. Recently, new molecular techniques have been introduced to deal with antimicrobial resistance. Researchers could knock out plasmid-mediated antibiotic resistance genes in order to prevent the spread of resistance. This review will discuss antibiotic resistance, CRISPR-Cas9 gene editing mechanism and its applications against bacteria itself, which will be an important method to prevent the clonal spread of resistant strains, providing a unique solution to the global problem.

Key words: CRISPR-Cas9, Gene editing, antibiotics, antimicrobials.

Introduction

1. History of the Antibiotics

Till the 20th century, deaths resulting from infectious diseases were serious problems. The antibiotic revolution started with the discovery of penicillin by Sir Alexander Fleming in 1928. The first penicillin was purified by Ernst Boris Chain and Howard Walter Florey in 1942, which then became world widely available in 1945 (Shama, 2008; Quinn, 2013). Deaths and amputations of soldiers during World War II were decreased significantly with the help of penicillin. While there were only 400 million units of penicillin available in 1943; following the World War II, companies achieved to produce 650 billion units (Raper, 1952). After the dramatic achievement of Fleming, discoveries of novel antibiotics against bacterial infections gained momentum. Over half of the antibiotics in use today were discovered and developed in between 1950 and 1960, which was termed "Golden Age" in terms of antibiotic therapies (Davies, 2006). Sulfonamides began to be used in the treatment in the 1930s onwards, including streptomycin in 1943, cephalosporins in 1945 (in use in 1967), chloramphenicol and tetracyclines in 1947, neomycin (first aminoglycoside), erythromycin, vancomycin, nalidixic acid (first quinolone) followed by fluoroquinolone derivatives followed by trimethoprim (co-trimoxazole with sulfamethoxazole) and other antibiotics in 1970 (Khardori, 2006; Adedeji, 2016). Following the clinical therapies with these antibiotic drugs, human health quality along with decreased mortality and morbidity rates related to infectious diseases was improved dramatically. Nevertheless, the fact that the pathogenic bacteria could gain antibiotic resistance wasn't foreseen (Aminov, 2009).

2. Development of Antibiotic Resistance

Antibiotic resistance is simply the ability to defeat an antibiotic that eliminates or stops the growth of the microorganism. Antibiotic resistance relates to microorganisms, antibiotics, the environment, and the patient or all of them. Researchers have reported that there have been serine beta-lactamases on plasmids for billions of years, conventional antibiotics-resistant surface bacteria strains in the 4-million-year-old cave ecosystem, and the discovery of the *vanA* resistance gene in 30,000-year-old Beringian permafrost sediments (Hall and Barlow, 2004; Allen *et al.*, 2009; Bhullar *et al.*, 2012). In fact, the antibiotic resistance is a natural phenomenon, which helped us to develop the antibiotics and derivatives for the clinical use. Antimicrobial resistance genes (resistome), which are naturally present in every region of the biosphere, are transferred to pathogen strains through mobile genetic elements (mobilome). In the last century, the main reason for the emergence of strains identified as multidrug-resistant, extended drug-resistant and resistant to all antibiotics is due to the resistome-mobilome cycle between pathogenic strains. Also, each of these is a pool of resistance genes in soil microbiota, food microbiota, animal microbiota, aquatic microbiota, wastewater microbiota, and human microbiota (Baquero *et al.*, 2008; Groer *et al.*, 2014; Martínez *et al.*, 2015).

At present, while the frequency of antibiotic resistance and the increase in resistance of clinically isolated pathogens is observed, the danger associated with the spread of "pan-resistant" strains identified as resistant to all classes of antibiotics is seen as alarming. Infectious diseases can't be treated due to antibiotic resistance, which develops due to unnecessary antibiotic use, wrong antibiotic selection, wrong dose and irrational use of antibiotics. The efficacy of antimicrobial drugs decreases due to

resistance and infectious diseases progress more seriously. This situation leads to a prolonged hospital stay, higher medical costs and increased mortality and morbidity rates (Fauci *et al*., 2005; Simpson, 2002; Slama *et al*., 2005; Ünal, 2005; Wright, 2007, 2010).

In 2011, epidemiological surveillance studies of the World Health Organization (WHO) revealed an increase in the resistance rates, and this resistance profile wasn't limited to a specific pathogen or region (WHO, 2011). 2014 WHO report warns about the resistance so that the antimicrobial resistance could spread worldwide till 2050, which may cause up to 10 million deaths per year unless taking any comprehensive counter-measures. This may result in up to 100 trillion USD an economic loss per year (O'Neill, 2016; Scarafile, 2016; Adli, 2018; Chokshi *et al*., 2019). Furthermore, the Organization for Economic Co-operation and Development (OECD) report suggests more intensive and expensive care of the hospitalized patients infected with the resistant bacteria, which may cost up to 40,000 USD (OECD, 2017). According to "Antibiotic Resistance Threats in the United States, 2019" report published by the Centers for Disease Control and Prevention (CDC), 2.8 million antibiotic-resistant infections per year are diagnosed in the U.S., causing deaths of more than 35,000 patients (CDC, 2019). Therefore, WHO suggested a worldwide action plan on the antimicrobial resistance in 2015, which aims the prevention and treatment of the antibiotic refractory infectious diseases with strengthening the surveillance network, changing the use of the antibiotics, and increasing sustainable investment in Research and Development studies and in the development of new antimicrobial drugs. In the absence of the novel antimicrobial therapies and/or effective antibiotics, medical interventions and diagnostics might not be possible in the future for prophylaxis (Adedeji, 2016; Tacconelli *et al*., 2018).

3. Alternative Therapies to Antibiotics

To overcome the problem of the antimicrobial resistance, there has been developed new therapeutic solutions including new schemes of antivirulence strategies, bacteriophage therapies, probiotics, therapeutic antibodies, synthetic inhibitor drugs specifically inhibiting resistance enzymes, bacterial efflux pumps, biofilm formation, fatty acid biosynthesis pathway, cell division, and amino acid metabolism in the antibiotic-resistant bacteria (Schimmel *et al*., 1998; Su and Honek, 2007; Lock and Harry, 2008; Lu and Collinsi, 2009; Njoroge and Sperandio, 2009; Kohanski *et al*., 2010). Bacterial genome modification techniques have also the potential to combat the infectious diseases (Krishnamurthy *et al*., 2016). Indeed, several programmable nuclease approaches such as Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) have been developed in studies for twenty years. However, the use of these methods has been obstructed by several disadvantages including the low efficiency, off-target effects, and a time-consuming labor force (Jinek *et al*., 2012; Nerys-Junior *et al*., 2018). Although these methods have been used successfully, none of them has been able to provide the speed, simplicity, high potential of modification and cost-effectiveness of CRISPR (Clustered regularly interspaced short palindromic repeat)-Cas (CRISPR associated) gene-editing system, which has been used frequently in genome modifications since 2012. These advantages of CRISPR-Cas technology allow it to be used in a wide range of research in biology. A new approach has emerged that enables researchers to directly manipulate cells for several gene editing approaches including knock-in of a single nucleotide variants to the gene-of-interest, insertion of a gene to the targeted deleted region of chromosomal regions. This technology is commonly referred to as "gene editing," "genome editing," or "genome engineering" (Gaj *et al*., 2013; Nemudryi *et al*., 2014; Gupta and Musunuru, 2014).

3.1. The CRISPR-Cas gene editing system

The CRISPR-Cas system is an important part of the adaptive immune system developed by bacteria and archaea against foreign DNA, such as plasmids or phage, which destroy the foreign genome. These systems are found in 95% of archaeal genomes and 48% of bacterial genomes. CRISPR systems are extremely diverse in terms of the diversity of Protospacer Adjacent Motif (PAM) and the number and type of Cas proteins. CRISPR-Cas mechanisms with the CRISPR region and the content of the Cas genes are classified into three main types (I, II and III) and 11 subtypes (I-A to I-F, II-A to II-C, and III-A to III-B) (Jiang and Doudna, 2015). Type II system is the most studied system and the mechanism is the best-illuminated system among these systems. The basic mechanism of the CRISPR-Cas9 system begins with the introduction of a foreign virus or plasmid DNA into the cell. Foreign nucleotides are recognized by Cas complex and are separated into approximately 30 base pairs in length and these fragments are inserted into the CRISPR sequence. This sequence contains small fragments of foreign virus or plasmid DNA that it has previously encountered. Foreign oligo DNA with the PAM sequence can be inserted into the guide RNA targeted site with repetitive genes. Cas proteins express and process the CRISPR region to produce CRISPR RNAs (crRNAs). In the Type II system, non-coding RNA trans-activating CRISPR RNA (tracrRNA) acts as a skeleton that binds crRNA with Cas9 and facilitates the conversion of precursor-crRNAs produced from CRISPR sequences into mature crRNAs. Using sequence homology, these crRNAs direct a Cas nuclease to the identified exogenous genetic material side next to the species PAM and breaks the targeted DNA region into fragments to form insertion-deletion mutation (Doudna and Charpentier, 2014; Ma *et al*., 2014; Nishimasu *et al*., 2014; Savić and Schwank, 2016).

The first CRISPRs were detected by Ishino *et al*. 1987 in *iap* gene of *Escherichia coli* genome. Ishino *et al*. discovered an orderly spaced short repeats located in the *iap* gene but their function had not been exactly figured out (Ishino *et al*., 1987; Ishino *et al*., 2018). In the following years, similar repeat sequences were determined in *Mycobacterium tuberculosis, Haloferax mediterranei, Methanocaldococcus jannaschii, Thermotoga maritima* and other bacteria - archaea (Sorek *et al*., 2008). It was reported at first by Barrangou *et al.* in 2007 that CRISPR sequences and Cas proteins function as allowing bacteria to recognize and destroy replicating genome of invading phages. Barrangou *et al.* found that *Streptococcus thermophilus* strains used in the production of yogurt and cheese were infected with phages and new-spacer DNA gains from the phage genome were discovered in the CRISPR locus. They also reported a correlation

between the number of spacer DNA and the phage resistance of the strain. Thus, CRISPR sequences and Cas proteins have proven to be an effective prokaryotic a nucleic-acid-based immune system against bacteriophage infection; defense against foreign genomes has been demonstrated by RNAs transcribed from the CRISPR locus (Barrangou *et al*., 2007; Barrangou and Marraffini, 2014). In 2008, Brouns *et al*. found that precursor RNAs (pre crRNA) (120 -180 bp) were first synthesized from the CRISPR locus in *E. coli* and cut into small mature RNAs (crRNA) (57 bp) by the activity of Cas genes (Brouns *et al*., 2008). In 2010, Garneau *et al*. reported that the gene, identified as cas9 encodes an enzyme capable of cleaving target DNA among Cas genes (Garneau *et al*., 2010). In 2012, after a very dramatic study by Emmanuelle Charpentier (Max-Planck) and Jennifer Doudna (UC Berkeley), the CRISPR-Cas system could be applied using gene-specific guide RNAs (gRNA) designed for gene modifications in prokaryotic cells. This technology has brought innovation in many areas as a gene regulation method that will mark the 21st century (Fichtner *et al*., 2014; Hsu *et al*., 2014; Sternberg, 2014).

According to Zion Market Research's report titled "Genome Editing Market by Technology (CRISPR, TALEN, ZFN, Antisense, and others), by Application (Cell Line Engineering, Genetic Engineering, and Others), and by End-User (Pharmaceutical & Biotechnological Companies, Academic and Research Institutes, and Contract Research Organizations): Global Industry Perspective, Comprehensive Analysis, and Forecast, 2017–2024", the global CRISPR genome regulation market was approximately 476.8 million dollars in 2017, it is estimated that it will grow by 36.8 percent between 2018 and 2024, and reach 4.3 billion dollars by the end of 2024 (Zion Market Research, 2018). It is estimated that CRISPR-based gene modifications will increasingly find applications in model systems. There are pioneering examples in which CRISPR-Cas9 systems were applied such as development of the cows that are *Tuberculosis*-resistant (Gao *et al*., 2017), treatment of mice by modifying the gene causing Duchenne muscular dystrophy (DMD) disease in mice uses the CRISPR method as in the production of maize and wheat strains resistant to drought and fungal pathogens. This technology continues to be used extensively in a wide range of fields such as medicine, agriculture, food, chemical, energy and environmental industries (Long *et al*., 2016; Nelson *et al*., 2016; Tabebordbar *et al*., 2016). In the following years, the CRISPR-Cas system will be used effectively and extensively in *ex-vivo* gene therapy studies in humans, and treatment of many cancer, autoimmune and chronic inflammatory diseases, genetic diseases will become possible. CRISPR technology will bring new therapeutic approach to the health field for many infectious diseases that aren't definitive treatment (Jinek *et al*., 2012; Gilbert *et al*., 2013; Hsu *et al*., 2014; Jiang and Doudna 2015; Peters *et al*., 2015; Khatodia *et al.,* 2016).

3.2 Bacterial CRISPR-Cas mechanisms engineered against the antibiotic-resistant bacteria

The CRISPR-Cas technology has been studied increasingly in the field of the biggest global health problem, namely the antimicrobial resistance. The development speed of new antibacterial agents decreased dramatically in the last twenty years. New antimicrobial strategies against antibiotic-resistant bacteria need to be developed to combating resistance. Gomaa *et al.* eliminated target-specific sequences from genomes in pure and mixed cultures with the CRISPR-Cas9 system. This targeting resulted in bacterial death because targeting the chromosomal genes results in bacterial killing. Researchers demonstrated that the CRISPR-Cas system resulted in considerable removal of the targeted strain in mixed cultures of bacteria while the other strain remained viable. This study demonstrated the advantageous of the CRISPR-Cas system over the phage therapies, antibiotics and other selective agents that is the capability of discriminating between different bacterial species and of targeting the genomes of the pathogens (Gomaa *et al.,* 2014).

Yosef *et al*. developed temperate phages to deliver the CRISPR-Cas system into antibiotic-resistant bacteria. Major problems to conventional phage therapy are the application of phage into infected tissue, bacterial resistance to phage, immunogenic response to phages and the large size of the phages. Yosef's study didn't require an application to host tissue. To eliminate the transfer of resistance genes from resistant strains to susceptible strains, the CRISPR-Cas system and a programmable DNA nuclease were used with phage. CRISPR-Cas system is programmed to antibiotic resistance-conferring plasmids and specific temperate phages. This system protected antibiotic-sensitive bacteria while allowing lytic phages to be programmed to kill antibiotic-resistant bacteria. Phage viruses containing the CRISPR-Cas system were antimicrobial by targeting the resistance gene. When lytic phage is applied, only antibiotic-resistant bacteria are targeted. When these bacteria were killed, antibiotics could be used to target the sensitive population. Phage viruses containing the CRISPR-Cas system were antimicrobial targeting the resistance gene *(bla_{ndm-1}* and *$bla_{ctx-M-15}$)*. When lytic phage is administered, only antibiotic-resistant bacteria are targeted. When these bacteria were killed, antibiotics could be used to target the sensitive population (Yosef *et al*., 2015).

Citorik *et al.* used two different systems, plasmid, and phagemid, to target the virulence gene and antibiotic resistance genes. They used the CRISPR-Cas9 system with plasmid and phagemid delivery systems to target the '*eae*' gene (for adhesion of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 to epithelial cells), beta-lactam (*bla_{NDM-1}* and *bla_{SHV-18}*) and quinolone (*gyrA*) resistance genes. In the first system plasmid conjugation and subsequent selective yield couldn't be obtained. Phagemid systems are plasmids encoding specific gene regions packaged with a phage (in this study M13 phage) capsid in a CRISPR-Cas targeting system. The effect of targeting on the *eae* virulence gene was examined by forming an infection model in *Galleria mellonella* larvae. The survival rate was significantly improved compared to the control groups. Targeting on the *bla_{NDM-1}* and *bla_{SHV-18}* genes by phagemids enabled the bacterial population to become susceptible to beta-lactams and showed a cytotoxic effect on bacteria carrying the quinolone resistance gene (Citorik *et al*., 2014).

Bikard and colleagues used a phagemid system. ΦNM1 phage for phagemid package, target kanamycin and methicillin resistance genes in *Staphylococcus aureus.* They also tested the efficacy of the CRISPR-Cas system using an *in-vivo* topical infection model in mice. After a skin infection with *S. aureus,* it was shown that phagemids reduced bacterial density from 50% to 11% within 24 hours compared to standard treatments such as topical mupirocin and streptomycin (200 mg/mouse) (Bikard *et al.*, 2014).

Wang and Nicholaou (2017) designed two CRISPR-Cas9 systems in Methicillin-resistant *Staphylococcus aureus* (MRSA) to target the promoter region of the *mecA* gene and suppress transcription of the resistance gene. When cefoxitin disc diffusion and oxacillin microdilution results were evaluated, changes in zone diameter and minimum inhibitory concentration were determined. The CRISPR system targeting the coding strand decreased antibiotic resistance and so was chosen for continued testing. The 77% decrease in gene expression wasn't enough to make MRSA clinically susceptible to beta-lactam antibiotics. The researchers planned to investigate the synergistic effects of plasmids designed for two CRISPR systems and then to perform the broth microdilution and *mecA* gene expression analysis (Wang and Nicholaou, 2017).

Kim *et al.* used the CRISPR-Cas9 system to resensitize the extended-spectrum beta-lactamase (ESBL)-secreting *Escherichia coli,* which possess plasmid-mediated antibiotic resistance genes that is used in horizontal gene transfer. The researchers targeted conserved sequences among the TEM- and SHV-type ESBL positive bacteria strains. The targeting of these genes with CRISPR-Cas9 provided the susceptibility of the bacteria to ampicillin and ceftazidime. The researchers described the change in bacterial resistance, which is an optimized strategy, namely Re-Sensitization to Antibiotics from Resistance (ReSAFR) (Kim *et al.,* 2016).

Ram *et al.* targeted the toxin genes of *S. aureus* on the pathogenicity island with the CRISPR-Cas9 system and aimed to reduce the virulence of bacteria and eliminate bactericidal effect and infection. For this purpose, they evaluated the efficacy of CRISPR technology in non-phage non-antibiotics called antibacterial drones (ABDs) and subcutaneous *Staphylococcus aureus* infections. *In-vivo* tests have shown that subcutaneous *S. aureus* abscess inhibits the development of infection by intraperitoneal administration of ABD particles and that the bactericidal effect of ABDs has been used for survival in mice (Ram *et al.*, 2018). At present, many kinds of research about phage cocktails, which often contain more than ten phage strains, are in the pre-clinical trial phases (Schmidt, 2019). Future research has shown that phage cocktails containing CRISPR-Cas can be used in therapy to target many pathogenic and drug-resistant bacteria species.

Conclusion

Instead of new antibiotics, the gene editing approaches has been developed to combat the insufficiency of potential antimicrobial therapeutic agents and the resistance to the conventional antibiotics. Recent innovations in synthetic biology have led to the development of new genome engineering tools including TALEN, ZFN, and CRISPR for manipulation of antibiotic-resistant microbial genomes using biotechnological applications such as phage therapy. With the development of the genetic engineering technologies, new antimicrobial products can be produced that specifically target virulent or antibiotic-resistant bacteria. The most important of these is the CRISPR-Cas mechanism, which is defined as the adaptive immune system of bacteria. CRISPR-based antimicrobials could be our newest defense against the antibiotic resistant bacteria such as multidrug resistant (MDR) strains.

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CRISPR-Cas9 based gene editing technologies in induced pluripotent stem cells

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Abstract: Recent advances in the field of induced Pluripotent Stem Cells (iPSC) have a crucial role in therapeutic research iPSCs are cells reprogrammed from somatic cells using different transcription factors. The unique features of iPSCs such as self-renewal and differentiation into various cell lines makes it a more advantageous candidate in stem cell technologies. By replacing the use of embryonic stem cells (ESCs), iPSCs usage overcome various ethical issues related to the use of embryos in research and clinics. Besides iPSC technology is a promising field for disease modelling and gene therapy as human-derived pluripotent stem cells are the ideal source of cells for autologous cell replacement. Furthermore for patients with single gene disease, it is vital to genetically correct the disease-causing mutation before cellular differentiation and transplantation. Hence, the emergence of the CRISPR-Cas9 system has a very revolutionary and significant role in the genome editing field. Compared to other gene editing technologies, it is relatively easy to implement and at a lower cost, it is possible to repair and modify the genetic composition. Therefore CRISPR-Cas9 is a promising tool by leading repair of patient-specific iPSCs and serving possible future autologous cellular treatments. In this review, the current approaches and gene editing technologies in iPSCs will be summarized.

Key words: CRISPR-Cas9, Gene editing, Induced Pluripotent Stem Cells (iPSC), Stem Cell.

1. Introduction

Human ESCs provide an important cell source for regenerative medicine due to their infinite self-renewal and ability to differentiate into all three germ layer cells. However, due to ethical problems, the use of ESCs is very limited. With the discovery of patient-specific iPSCs, both immunogenic problems related to transplantation of allogeneic cells and ethical concerns have been solved (Takahashi and Yamanaka, 2006; Li et al., 2018). As cellular mechanisms vary in different species in health and disease states, human ESC and human iPSC provides an essential and promising technology by generating peculiar lineage committed cells for clinical studies. Unlike the production of human ESCs that cause embryo destruction, iPSCs can be produced on request from patients, and this condition is ethically preferred. Therefore, iPSCs have been the focus of interest for disease modelling, regenerative medicine, drug screening, and biomedical research. Since the development of iPSC technology, a variety of patient-specific iPSC lines have been produced in the investigation of hereditary diseases such as neurodegenerative, metabolic and cardiac. (Jehuda et al., 2018). In the modelling of hereditary genetic diseases, any cell type obtainable from patients can be used for iPSC derivative since they all carry disease-causing mutations. The major criteria in the cell type selection while modelling disease are availability, tissue accessibility and ease of tissue processing and culture. Skin fibroblasts and peripheral blood (PB) cells are the two most predominantly used cell sources for this purpose. In addition, bone marrow (BM) stromal cells, keratinocytes, adipocytes, urinary epithelial cells collected from urine samples and amniotic fluid cells are also used (Georgomanoli and Papapetrou, 2019).

Human iPSCs show great similarity to ESCs in terms of phenotype and culture characteristics, and unlike ESCs, embryo use during the derivation of iPSCs is out of the question. Another advantage of the iPSC lines is that these cells are derived from the patient and thus providing unlimited access to disease-specific differentiated cells for research such as disease modeling, drug screening (Merkert and Martin 2016).

2. Induced Pluripotent Stem Cells (iPSCs)

In 2006, Takahashi and Yamanaka discovered that mouse skin fibroblasts could be programmed into iPSC using a mix of pluripotence transcription factors, and these results significantly changed the scope of stem cell research. A year later, James Thomson and George Daley, together with the same researchers, succeeded in transforming human fibroblasts into human iPSCs. Afterwards pluripotent reprogramming has been demonstrated in various somatic cell types (Young et al., 2012).

iPSCs are cells that are converted from somatic cells to pluripotent stem cells (PSCs) by reprogramming factors (Oct3/4, SOX2, c-Myc and KLF4) and resemble ESCs in morphology, molecular and functional aspects (**Table 1**) (Omole and Fakoya, 2018). Theoretically, any somatic cell type can be reprogrammed to acquire pluripotent properties (Takahashi and Yamanaka, 2006; Georgomanoli and Papapetrou, 2019; Ruiz et al., 2011).

Since its inception in 2006, iPSC technology has evolved rapidly. Initially, differentiation of iPSCs from other somatic cells was carried out by using programming factors and by integrating viral vectors (Kiskinis and Eggan, 2010). However, the possibility of insertional mutagenesis due to integration of these iPSCs into the host genome has raised concerns in clinical practice (Saha and Jaenisch, 2009; Shi et al., 2017). Later, non-integrating methods such as episomal DNA, adenovirus, recombinant proteins, synthetically modified mRNAs, microRNAs have been developed to make the iPSCs clinically viable (Shi et al., 2017). Among these approaches, particularly episomal DNAs, synthetic mRNAs and sendai virus, are more widely applied because of their greater efficiency and relatively easier applicability. The human iPSCs created using these non-integrating approaches are more suitable for clinical applications and constitute a disease-associated cellular resource. (Shao and Wu 2010; Shi et al., 2017).

Table1. Characterizationof iPSCs.

iPSC	**Pluripotency markers** -Alkaline phosphatase assay (as a live marker) -Increase levels of pluripotency proteins such as Oct4, Nanog, SSEA3/4, TRA-1-60, and TRA-1-81
	Morphology -Flat, cobblestone-like cells, ES like morphology -Tightly packed colonies with sharp edges
	Epigenetic analyses -DNA methylation of lineage-committed genes -DNA demethylation of key pluripotency genes like Oct4, Sox2, Nanog
	Genetic analyses -Diploid karyotype -Transgene silencing after reprogramming
	Differentiation potential -Teratoma formation—can form ectoderm, mesoderm, and endoderm, the three germ layers -Embryoid body formation—can form ectoderm, mesoderm, and endoderm, the three germ layers

Since 2007, the rapidly evolving human iPSC technology has launched an exciting new era in regenerative medicine such as stem cell biology, disease modelling and drug discovery (**Figure 1**). Animal models play an important role in the investigation of disease mechanism. However, because of the fundamental developmental, biochemical and physiological differences between mice and humans, the use of human cells to better understand disease mechanisms is important to prevent failures (Kiskinis and Eggan, 2010; Shi et al., 2017).

Human pluripotent stem cells have the potential to produce all tissues in the body. This enables the researchers to reach the patient-based biomaterial in order to understand the mechanism of the disease and to conduct therapeutic research. The creation of specific disease models by programming of iPSCs involves in two important stages. First, programming the iPSCs from the patient's somatic cells and then differentiating the iPSCs into the affected cell types (Saha and Jaenisch, 2009).

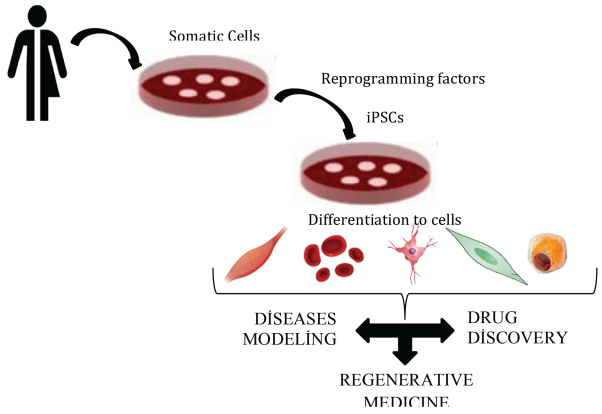

Figure 1. Reprogrammed iPSCs from somatic cells

3. Genome editing and CRISPR-Cas9

Genome regulation technology has been developing rapidly in recent years. In particular, four nuclease-based platforms, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases and most recently, clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein 9 (Cas9) (CRISPR-Cas9) systems have made significant improvements. While previous approaches are not preferred due to error rate and high non-target effect, the CRISPR-Cas9 system has been reported as a more accurate and effective method for regulating the human genome (Cai et al., 2018).

Firstly in 1987, CRISPR has been discovered by Ishino et al. in *E. coli* while studying the iap gene responsible for isoenzyme conversion of alkaline phosphatase , however its exact function is not described. Later, Mojica et al. described repeat sequences in the genome of different prokaryotes and reported that similar repeat sequences exist even in distant phylogenetic groups. It has been reported that there are short repetitive sequences at regular intervals, and these repetitive sequences are interrupted by fixed length intervening sequences at regular intervals. Researchers have described these sequences as Short Regularly Spaced Repeats (SRSRs) (Mojica et al., 2000). In 2002, Jansen et al.also studied a new family of repetitive DNA sequences found in Archaea and Bacteria, but not in eukaryotes or viruses, and called these sequences regularly intermittent short palindromic repeats (CRISPR) and also identified 4 different cas genes associated with CRISPR regions. In 2005, various research groups reported that CRISPR was observed in prokaryotes and supported the acquired immunity to viruses (Bolotin et al., 2005; Pourcel et al.,

2005). Research in CRISPR technology has accelerated in the following years.

The CRISPR–Cas system, originally described as an adaptive immune system in bacteria and archaea, is now designed as RNA-directed endonucleases for genome regulation, enabling rapid, inexpensive and relatively easy correction of errors in the genome. (Ma et al., 2014; Redman et al., 2016). In this technology, programmable nucleases, similar to DNA restriction enzymes, cuts DNA double strands in the region where genome editing is desired. While these double strand cuts are repaired by homology directed repair (HDR) or non-homologous end-joining (NHEJ) cell DNA repair mechanisms, different modifications occur in the genome depending on which repair mechanism is used by the cell. (Cox et al., 2015).

CRISPR-Cas9 has an important role in the fight against infections such as hepatitis B virus and human papillomaviruses, in monogenic diseases in model organisms and in correcting target mutations. Recently, significant progress has been made in this area. One of the most exciting applications of CRISPR-Cas9 is its use for the treatment of genetic diseases caused by single gene mutations such as Duchenne muscle dystrophy (DMD), cystic fibrosis (CF) and hemoglobinopathies (Dai et al., 2016; Redman et al., 2016). There are several studies related to the in vivo genome editing for the repair of DMD-causing mutations in the dystrophic mouse model published in Science. These studies demonstrated the potential of gene editing with CRISPR-Cas9 in the treatment of DMD as the recovery of dystrophin expression increased muscle strength (Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016). In another study, it was reported that the target mutation in the organoid system formed by intestinal stem cells isolated from cystic fibrosis patients was corrected by using homologous recombination with CRISPR-Cas9 technology (Schwank et al., 2013).

Gene editing has recently emerged as a promising way to treat hematological diseases in particular. One of these, sickle cell disease (SCD), is the result of a single nucleotide polymorphism in the β-globin gene (HBB). It is a recessive genetic disease characterized by a decrease in the red blood cells in the blood as a result of the deterioration of hemoglobin structure. As a result, when tissues and organs do not get enough oxygen, they become damaged, anemia occurs, the body becomes susceptible to infections and may cause premature death. Various studies have been carried out on CRISPR-Cas9 technology for ex vivo gene editing and optimization of human HSPCs in hematological diseases such as SCD (Hendel et al., 2015; DeWitt et al., 2016). Hendel et al. have shown that chemically synthesized sgRNAs in human primary T cells and CD34+ hematopoietic stem and progenitor cells (HSPCs) increase the efficiency of genome editing. They reported that chemically synthesized and modified sgRNAs have advantages over expressed or in vitro transcribed sgRNAs and have lower cytotoxicity in primary cells than DNA plasmid-based systems (Hendel et al., 2015).

Although the CRISPR-Cas9 system has shown great promise for gene editing and treatment approaches, several factors affecting its efficacy should be considered, especially when used for in vivo human gene therapy. Of these, target site selection and sgRNA design have been shown to be more difficult and important than originally thought. In addition to the design of the SgRNA, the off-target rate and increasing the specificity are also important factors in the success of the CRISPR-Cas9 system. It has also been reported that the incidence of HDR-mediated repair in DNA double-strand breaks (DSB) is extremely low in mammalian cells and inhibitors such as Scr7 are used to increase HDR-mediated gene editing. While Scr7 increases the efficiency of HDR by 19 times, it has been reported that these and other inhibitors may have toxic effects on host cells. At the same time, different PAM sequences and Cas9 protein which show variable activity and identified from different species, can enhance gene editing efficiency for a specific target sequence and should be considered as an important part of the gene editing system (Lino et al., 2018).

4. Induced Pluripotent Stem Cells and CRISPR-Cas9

PSCs are endless self-renewing cells and can be transformed into many different cells. Due to these properties, it is thought that it can be used in understanding and treating the mechanism of many degenerative and genetic diseases. Particularly in regenerative medicine, they are important tools for establishing patient-specific disease models.

iPSCs are also important for the prevention of immunological reactions, especially in the treatment of transplantation, because of patients originate from their own cells. These cells have the same characteristics as the cells from which they originate, and as they carry the same genetic mutations as the patient, they have an important role in understanding the mechanisms of certain diseases and developing a patient-specific treatment approach.

Genome regulation in iPSCs is very important for investigating genetic, molecular and cellular mechanisms associated with hereditary diseases. However, CRISPR-Cas9 has significant potential for patient-specific therapeutic regenerative medicine. CRISPR and CRISPR-associated (Cas) genes were first discovered in E. coli and S. epidermidis as an adaptive immune system to protect bacteria from bacteriophage assault (Jehuda et al., 2018).

Human pluripotent stem cells are ideal candidates for new cell-based regenerative repair due to two important properties: 1) renew themselves indefinitely and 2) potentially differentiate into any cell type (Angelos and Kaufman, 2015). At the same time, being of human origin, easier to obtain, expandability, ability to differentiate into three different germ layer cells are important in terms of not causing ethical problems compared to human ESCs. However, there is the potential for personalized therapeutic development using patient-specific iPSCs. Thus, recent advances in CRISPR-Cas9 gene editing technology, in particular, allow the rapid creation of genetically defined human iPSC-based disease models (Shi et al., 2017).

ZFNs and TALENs were administered for gene manipulation of human iPS cells. However, since both ZFNs and TALENs need the design of DNA-binding proteins and the construction of

complex plasmids for expression of these proteins, these methods are costly, time-consuming, and not easily applicable (Horii et al., 2013).

Compared with ZFNs and TALENs, CRISPR-based genome regulation has been reported to have some advantages in practice. First, single guide (sg) RNAs are easier to design and produce faster than protein-based DNA targeting motifs used in ZFNs and TALENs. At the same time, CRISPR-based genome editing is more specific and more efficient than other genome editing tools. In addition, CRISPR-based genome editing is more potent for multiplex gene editing and can add or remove multiple genes simultaneously using different sgRNAs. Therefore, the CRISPR-Cas9 system has been shown to be a powerful and more useful platform for studying polygenic disease mechanisms, setting new therapeutic targets and establishing disease models (Cai et al., 2018).

Extensive improvements to the CRISPR-Cas9 system have made numerous strides in increasing the specificity and effectiveness of the CRISPR-Cas9 mediated genome-editing platform. The main challenge of the genome editing field could be overcomed by the reduction of off-target impacts with the help of several innovative software programs that allow the prediction of off-target cleavage site (Cai et al., 2018).

First studies at human pluripotent stem cells with CRISPR-Cas9 have focused on the correction of patient-derived iPSCs, especially in hematologic patients.In addition, CRISPR-Cas9 mediated gene editing studies for many diseases have been performed in iPSCs (**Table 2**).

Various CRISPR-Cas9 applications have been performed to repair β-thalassemia-causing mutations in patient-induced iPSCs in β-thalassemia, which is one of the most common hereditary blood diseases (Xu et al., 2015; Song et al., 2015). In sickle cell disease (SCD), another blood disease, the point mutation in the HBB allele in patient-specific iPSCs has been shown to be effectively corrected by CRISPR-Cas9 mediated genome editing, and normal HBB proteins have been reported to be expressed in erythrocytes after hematopoietic differentiation of edited iPSCs (Huang et al., 2015).

In the case of compound heterozygosity in which HbE and β-thalassemia coexist (HbE/β-thalassemia), it is manifested by anemia requiring red cell transfusion in the first year of life, similar to homozygous β-thalassemia. Although hematopoietic stem cell transplantation is the only treatment option, only allogeneic transplantation is recommended because of the lack of appropriate HLA compatible donors and the morbidity, mortality and immunological complications associated with the transplant. In the present study, it was reported that HbE mutation was successfully repaired in patient-derived iPSCs carrying HbE/β-thalassemic compound heterozygote mutation using gRNA and ssODN template designed to recognize HbE mutation. Consequently, it has shown that genetic correction of HbE mutation in an allele is sufficient to restore HBB protein expression (Wattanapanitch et al., 2018).

Table 2. CRISPR-Cas9 Mediated Gene Editing Studies in Induced Pluripotent Stem Cells.

Disease	CRISPR-Cas9 studies	Reference
Fabry Disease (FD)	Gene editing technology was applied to patient-induced iPSC.	Birket et al., 2019
Immunodeficiency, centromeric region instability, facial anomalies syndrome (ICF) syndrome	The iPS cell model was generated and mutated iPS cells were obtained in both DNA methyltransferase3B (DNMT3B) alleles of transfected clones.	Horii et al., 2013
Chronic granulomatous disease (CGD)	A high level of gene correction was reported using CRISPR-Cas9 from iPS cell lines derived from a patient with single point mutation (T> G) at the end of intron 1 in the CYBB gene.	Flynn et al., 2015
Amyotrophic lateral sclerosis (ALS)	Corrected ALS iPSCs were generated in the pluripotent stem cells differentiated from fibroblasts of ALS patients using the CRISPR-Cas9 system.	Wang et al., 2017
Hemophilia B (HB)	The approach for HB gene therapy was developed using the CRISPR-Cas9 system in patient-derived iPSC.	Morishige et al., 2019
Enhanced S-cone Syndrome (ESCS) associated with NR2E3	A repair strategy for CRISPR-based homology was developed and corrected the NR2E3 mutation that caused two different diseases in patient-induced pluripotent stem cells (iPSC) of two affected individuals.	Bohrer et al., 2019
β-thalassemia	An effective approach has been developed for the generation of patient-derived pluripotent stem cells (iPSCs) and the correction of disease-causing HBB mutations with CRISPR / Cas9 technology.	Xie et al., 2014
β-thalassemia	Patient-derived iPSCs carrying the IVS2-654 (C> T) mutation in the HBB gene were successfully repaired by CRISPR-Cas9 and ssODN-mediated HDR repair.	Xiong et al., 2019
Dystrophic epidermolysis bullosa (DEB)	Efficient gene editing was achieved by repair of patient-induced induced pluripotent stem cells (iPSCs) with homology to the CRISPR-Cas9 gRNA ribonucleoprotein complex system.	Jacków et al., 2019
Haemoglobin (Hb) H-constant spring (CS) alpha thalassaemia	A therapeutic approach is presented as a result of CRISPR-Cas9 based gene correction of patient-specific induced pluripotent stem cells (iPSCs) and cell transplantation.	Yingjun et al., 2019
Primary hyperoxaluria type 1 (PH1)	There is evidence that CRISPR-Cas9 nuclease-mediated gene targeting in patient-specific iPSCs is an effective strategy for producing functionally corrected hepatocytes without extra-target inserts.	Estève et al., 2019
Hemophilia A (HA)	Reparation via CRISPR-Cas9 has been reported in patient-derived induced pluripotent stem cells (iPSCs).	Park et al., 2019

Fanconi anemia (FA) is an another disease in which studies for treatment with CRISPR-Cas9 in iPSCs occur. FA is a complex disease caused by mutations in FANC genes. Sequence mutations of these genes are characterized by developmental abnormalities and bone marrow insufficiency. Although hematopoietic cell transplantation (HCT) is the only curative treatment option for

fatal bone marrow symptoms of the disease, there are risks associated with transplantation. Osborn et al. reported that iPSCs obtained by reprogramming fibroblasts of patients with mutations in the FANCI gene were repaired using Cas9 nicase, and this approach may be potential for patient-specific treatment (Osborn et al., 2016).

Despite advances in iPSC and gene editing technologies, there are still many challenges needed to be overcomed. One of the most important problems is that human cells prefer an indefinite NHEJ repair mechanism instead of the more precise HDR repair mechanism using an exogenous repair pattern to repair the DSB. The preferred NHEJ-mediated repair mechanism of cells usually results in insertions and deletions in gene regulation. The small molecules used for this cause inhibition of the NHEJ repair mechanism and are directed to the cell HDR repair mechanism. However, further studies are needed regarding the consequences of this (Hockemeyer and Jaenisch, 2016).

5. Conclusion

Recent development in DNA sequencing technologies contribute to the identification of numerous candidate loci associated with the diseases. Accordingly, there is a need for simple, strong disease models that can be applied to understand the functionality of genetic changes. The coexistence of iPSC and genome editing technologies is crucial because it allows the examination of some diseases in the human cellular system. The dual usage of iPSCs and genome regulation technologies will, certainly, ensure us with more information about disease mechanisms and therapeutic targets, and will allow the characterization of genetic deviations that cause certain diseases. It is also important that iPSCs originate from patients' somatic cells, particularly in the prevention of immunological reactions in the treatment of transplantation.

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