Investigating the Role Of CRISPR In Treating Hematopoietic Malignancies

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Abstract

Since its discovery, CRISPR has accelerated progress in the understanding and treatment of many genetic diseases, with an efficiency and accuracy that greatly surpasses those of previous methods of genome editing. CRISPR has advanced the field of leukemia research and therapy because of the ease with which hematopoietic cells can be obtained, studied, edited, and transfused into the patient as treatment. CRISPR has been used to screen for therapeutic targets in leukemia, and to generate a wide variety of animal and cellular models to facilitate leukemia research and drug development. In the clinical setting, CRISPR has been applied to leukemia treatment through immunotherapy, and has shown specifically remarkable results when used to edit T-cells for CAR T-cell therapy. CRISPR can also be used in leukemia therapy if employed for the genetic editing of hematopoietic stem cells for autologous transplantation. This review aims to discuss the role of CRISPR in the fight against leukemia and focuses on the assessment of the performance of CRISPR as a gene editing tool in leukemia research and therapy. It also aims to bring forth the limitations regarding the use of CRISPR and identifies areas where there is scope for improvement. Further, the review also aims to address ethical and safety concerns associated with the use of CRISPR as a gene editing tool and concludes with a final evaluation of its use in leukemia research and therapy.

1 An Introduction to CRISPR and Leukemia

Genome editing is defined as the ability to modify an organism's DNA sequence, and thus customize its genetic makeup. The first targeted genome editing was done in yeast and mice in the 1980's, and since then has been used to advance the understanding, diagnosis, and treatment of various genetic diseases, including malignancies like leukemia. Gene editing is performed by using enzymes, specifically nucleases, to induce double strand breaks (DSBs) in the gene of

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interest. These DSBs, once induced, cause the broken ends to be repaired using either homologous recombination or non-homologous end joining (NHEJ). During homologous recombination, the DSBs are repaired by resynthesis using another DNA sequence as a template, and during NHEJ the ends are joined together precisely, and sometimes a few base pairs are inserted or deleted. These mutations often lead to inactivation of a gene (Carroll, 2020).

Today, there are three powerful nucleases that can be used for genome editing. These are zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR). Out of these, CRISPR is the most precise and inexpensive way to perform gene editing.

A recently developed tool for genome editing, CRISPR is derived from bacterial and archaeal cells, and has been modified for use in human cells. In bacterial and archaeal cells, CRISPR, along with CRISPR-associated nucleases (Cas), is an RNA-guided defense mechanism deployed by the cells to

protect themselves from invading viruses. This defense mechanism is characterized by the incorporation of short sequences of DNA obtained from the invading virus into the host's own DNA, at a site in the host's genome known as the CRISPR locus. The CRISPR locus is a specialized region of DNA that contains nucleotide repeats and spacers. Repeated sequences of nucleotides are distributed among the CRISPR locus, and spacers are shorter sequences present among these repeats. In bacterial cells, the spacers comprise the genetic material obtained from the invading viruses. These sequences are transcribed into CRISPR-RNA (crRNA) that guides the destruction of the invading genetic material, should the host cell encounter the same virus again (Vidyasagar, 2020). In the bacterial genome, there is also a sequence of genes known as CRISPR-associated, or "Cas" genes. These genes make Cas proteins which are helicases and nucleases and help in destroying the invading genetic material.

The function of CRISPR as a prokaryotic defense mechanism was first demonstrated in 2007, in the bacterium Streptococcus thermophilus (Ishino et al., 2020). An incorporation of a phage sequence in the spacer region of the CRISPR locus in S. thermophilus made it resistant to the corresponding phage. This resistance disappeared when the sequence was removed from the CRISPR locus, demonstrating that it was the presence of those spacers in the CRISPR locus that was responsible for the resistance of bacteria towards certain viruses.

The ability of the CRISPR unit to function in a heterologous (derived from a different species that the recipient) manner in other species was demonstrated in 2011, by Siksnys and colleagues. The CRISRP locus from S. thermophilus was successfully cloned and expressed in E. coli, which suggested that CRISPR systems are self-contained units that can express themselves in cells from different species. CRISPR was formally harnessed for genome editing two years later, in 2013, by Feng Zhang (Broad Institute, 2020).

1.1 The Mechanism of CRISPR

The components of the CRISPR system include a guide RNA (gRNA or sgRNA) sequence that specifically binds to a 20-base pair sequence of interest in the target genome, and the Cas enzyme, which is the endonuclease that induces the DSB in the gene of interest (refer to Figure 1). A guide RNA molecule is a combination of CRISPR RNA (crRNA), which is about 17-20 base pairs long and binds to the target DNA using standard Watson-Crick base pairing rules, as well as the tracer RNA, which holds the CRISPR RNA in place and acts as a binding scaffold for the Cas endonuclease ("Synthego, 2020). The target DNA of the Cas protein can thus be modified based on the nucleotide sequence present as the crRNA portion of the guide RNA (Addgene, 2020). The target DNA must be followed by a dinucleotide-containing protospacer adjacent motif (PAM) sequence upstream from the gRNA binding site, for the endonuclease to recognize and cleave the DNA sequence. The PAM is an important part of DNA binding, and studies have shown that Cas9 is unable to entirely recognize the target sequence complimentary to the gRNA in its absence. Another crucial component of CRISPR gene editing technology is the different Cas proteins which have different bacterial cell origin. Out of these different proteins, the Cas9 protein is the most widely used by scientists for research, which is isolated from Streptococcus pyogenes. In the case of Cas9, the protospacer adjacent motif sequence is NGG (New Scientist, 2020). Today there are three known types of CRISPR-Cas9 systems, of which the type II is the most well- known and applied in genome editing. This is because type II CRISPR- Cas9 only requires a single protein for DNA recognition and cleavage (De Masi et al., 2020).

As mentioned above, after using CRISPR-Cas9 to induce a DSB in the genome, the cell's DNA r e p a i r m e c h a n i s m attempts to repair the broken ends using either h o m o l o g o u s recombination or non- homologous end joining. To inactivate a gene, NHEJ is best used, and to insert a gene into the target DNA sequence, homologous recombination takes place, using a target donor DNA sequence as a template.

There are multiple methods that can be used to deliver the CRISPR-Cas complex to the target cells. These include viral, non-viral, and physical methods. Viral-mediated delivery is done using vectors such as adenoviral vectors, adeno-associated viruses (AAVs), and lentiviral vectors. These viruses recognize and enter the target cell, where they release the viral genome for replication, which contains programmed genome editing nucleases. After replication, the reproduced virions leave the cell and go on to infect neighboring cells. This continues in a cycle, until a large number of cells possess the nucleases (Chandrasekaran, Song, Kim and Ramakrishna, 2020). Non-viral vectors include polymeric materials, cell-penetrating peptides, liposomes, and cationic nanocarriers. The biggest advantage of non-viral vectors is their non-hazardous nature, as well as their capability to accommodate large components for delivery (Chandrasekaran, Song, Kim and Ramakrishna, 2020).

The main physical methods that allow for the delivery of the CRISPR-Cas

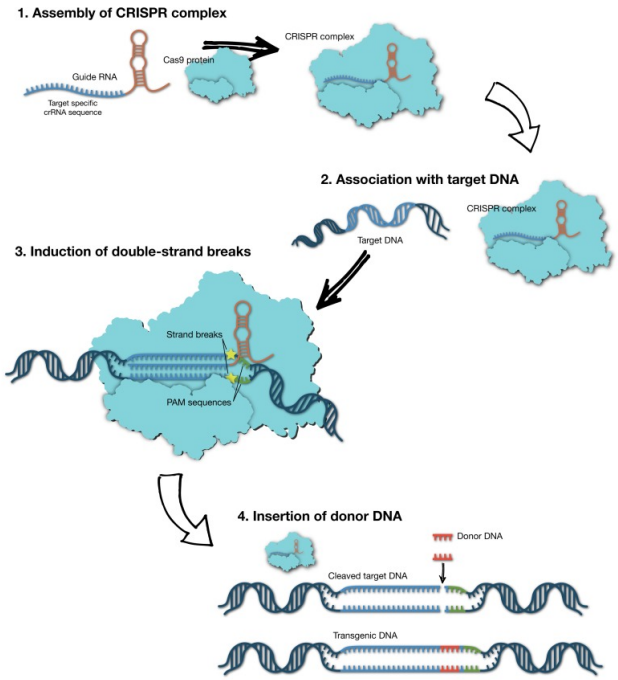

Figure 1: Figure 1: CRISPR mechanism during gene editing (Costa et al., 2017)

complex into target cells are electroporation and microinjection. For delivery by microinjection, a needle with diameter 0.5 to 20 micro- meters is use for insertion into embryos, aided by special microscopes and micro-manipulators. Alternatively, electroporation uses electric pulses to generate pores on the cells, which allows for the entry of the CRISPR complex into the cell (Chandrasekaran, Song, Kim and Ramakrishna, 2020).

Plausibly, the biggest advantage of CRISPR over other genome editing techniques is the simplicity and efficiency with which it works. Since the specificity of the CRISPR-Cas system is defined by RNA-DNA complementarity, there is no need for the engineering of proteins to bind to specific DNA sequences in the target genome, which is required in the case of ZFNs and TALENS. This does not only make the process inexpensive, but also relatively faster than other gene editing techniques. Additionally, CRISPR- Cas9 has shown to be able to target heterochromatin sequences too, which are DNase-inaccessible locations, as well as the ability to cleave highly methylated regions of DNA. Another factor which lends CRISPR the upper hand when it comes to gene editing is that multiple variants of Cas proteins allow for a flexible selection of target DNA (Meštrović, 2020).

In the last few years, the CRISPR-Cas9 genome editing system has been used for a number of purposes such as to enhance disease understanding, further drug

discovery, and discover new and improved treatments to previously untreatable diseases. Some areas of research which have benefitted from this are those dealing with blood disorders such as beta thalassemia and sickle cell anemia, with genetic diseases such as cystic fibrosis and Huntington's diseases, with HIV-AIDS, and with various forms of cancer. One of these where CRISPR is an extremely useful tool is leukemia, or malignancies of the blood (Fernández, 2020). CRISPR can be used in a plethora of ways to aid leukemia research and treatment, many of which are widely prevalent already, such as in the creation of animal models, the identification of therapeutic genome targets, and to aid immunotherapy and in-vivo gene editing as therapies for leukemia. CRISPR-mediated gene editing has been particularly useful in leukemia research and therapy for two main reasons: (1) the genetic nature of leukemia; and (2) the accessible nature of blood bone marrow cells, in particular the ease with which hematopoietic stem cells can be obtained from and transfused back to patients suffering from leukemia.

1.2 Etiology of Leukemia

Cancer is the second largest cause of death worldwide and is a major economic as well as social cause for worry. Of the many forms of cancer, one of the most lethal today is leukemia, or blood cancer.

Cancer is essentially a genetic disease. It is caused by mutations in the genome of a cell, that accelerate cell division or inhibit normal growth controls on the cell. These mutations, which usually result in the activation of oncogenes or the inactivation of tumor suppressor genes, accumulate over time, and as these cells grow and divide, they begin to increasingly differ from normalcy. As a result, these cells acquire an accelerated ability to proliferate, causing them to rapidly divide and eventually stop responding to signals that regulate their growth and division. This large number of mutations in a cancer cell's genome would make it an ideal target for apoptosis, but cancer cells often manage to escape even

programmed cell death, which is a way that cells "commit suicide" by activating an intracellular death program. This leads to the accumulation of many dysfunctional cells that develop into a cancer (Nature Education, 2014).

Leukemia is a cancer of the blood and the bone marrow. The bone marrow contains hematopoietic stem cells, which are multi-potent cells that give rise to different kinds of blood cells. These hematopoietic stem cells further differentiate to form either common myeloid progenitor cells (CMP cells) or common lymphoid progenitor cells (CLP cells). The CMP cells give rise to megakaryocytes (which later differentiate into thrombocytes or platelets), erythrocytes (RBCs), and myeloblasts (which later differentiate into basophils, neutrophils, eosinophils, and monocytes). The CLP cells give rise to lymphocytes (small lymphocytes and large, granular lymphocytes) (The Human Protein Atlas, 2020). Genetic abnormalities in this chain of differentiation (refer to Figure 2) of stem cells into blood cells lead to blood disorders, one of them being leukemia, which is a cancer in which an abnormally rapid production of dysfunctional leukocytes

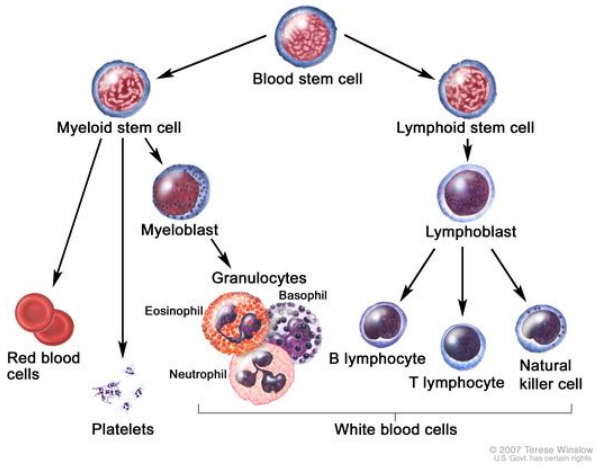

Figure 2: Figure 2: The differentiation of blood cells from HSPCs (NCI Dictionary of Cancer Terms, 2020)

takes place. Malignant leukocytes are immature, unable to fight infections in the body, and impair the production of erythrocytes in the bone marrow. Based on the affected progenitor cell, leukemias are either lymphocytic (also known as lymphoid or lymphoblastic leukemia) or myelogenous (also known as myeloid or myelocytic). They can also be either chronic or acute. Chronic leukemias progress slowly over time, unlike acute leukemias, which demand immediate attention and treatment (Leukemia, 2020). Consequently, leukemias are divided into four different sub-types: Acute lymphocytic leukemia (ALL), Acute myelogenous leukemia (AML), Chronic lymphocytic leukemia (CLL) and Chronic myelogenous leukemia (CML) (Labpedia.net, 2020).

An average of about 2.6 percent of all cases of cancer diagnosed worldwide are cases of leukemia (World Cancer Research Fund, 2020). As of 2020, 9.4 percent of all deaths caused by cancer worldwide are estimated to be caused by forms of leukemia (Leukemia and Lymphoma Society, 2020). In accordance with these statistics, while the fraction of cancer instances that are leukemias is not very high, the mortalities caused by leukemias make up a much larger fraction of mortalities caused by all forms of cancer in total. However, in the past few decades, leukemia, of all cancer types, has seen the largest improvement in survival rates. The 5-year survival rates have increased from about 34.0 percent in 1975-1977 to about 61.4 percent today. This increase in the survival rate of patients diagnosed with leukemia can be attributed to the advances in leukemia research, which have enabled the discovery of more specific, less toxic, and more effective therapies. The main forms of therapy have been chemotherapy, radiation therapy, and surgery, but these often have harmful side effects. While the toxicity of chemotherapy increases a patient's lifespan, it unfortunately also decreases their quality of life to a great extent (Braun et al., 1997).

A hopeful forefront to leukemia treatment, still in a stage of infancy, is gene therapy, particularly using CRISPR. This paper investigates and evaluates the role that CRISPR has played in the past, and could play in the future, of leukemia treatment.

Since cancers are essentially of genetic origin, genome editing has become critical in the treatment of various forms of cancer, as well as leukemia. At present, CRISPR-Cas systems are being used to investigate genetic mechanisms in almost all forefronts of cancer, from prevention to prognosis and treatment. An example of this is the application of CRISPR in immunotherapy. Immunotherapy has recently emerged as a promising way of cancer treatment, by harnessing the power of a patient's own immune system to eliminate tumor cells, a mechanism which will be elaborated upon later in this review.

Throughout the past few years, CRISPR has been used to advance our understanding of leukemia in a number of ways, for use in both the laboratory as well as therapeutically. In the laboratory, CRISPR is used to identify additional therapeutic targets in the leukemia genome, to generate animal and cellular models to study leukemia, as well as to generate accurate models for the testing of newly developed leukemia drugs. Therapeutically, CRISPR is used in two broad ways to treat leukemia- through its application in immunotherapy, and in the process of gene editing of cells for autologous transplantation. This review aims to elaborate on these methods, as well as their advantages, disadvantages, and scope for improvement, throughout.

2 Using CRISPR to Understand and Treat Leukemia

This section elaborates upon the different ways in which CRISPR aids leukemia research and therapy. First, the application of CRISPR in leukemia research and drug development is discussed, followed by a discussion on the use of CRISPR in therapeutic clinical procedures to treat leukemia.

2.1 CRISPR in Leukemia Research and Drug Development

The process of drug discovery (or drug development), in which potential compounds are screened and evaluated for therapeutic use, has led to the development of safe and effective drugs for many diseases, including cancers like leukemia. The assessment of many candidate compounds is a tedious and costly process. As a result, only a very small number of compounds make it to the market as therapeutic drugs. The entire process may take up to as long as 15 years, and can cost over cost over 1 billion dollars (Thorne, 2019). To eliminate such barriers of time and cost in the process of drug discovery, using inexpensive and efficient technologies like CRISPR is key.

The drug discovery process comprises several steps before new therapeutics can be approved for treatment. The main steps common to most therapeutic drug discovery processes, before the drugs are approved for clinical trials, are

target identification and validation, compound screening, hit validation, and lead drug candidate optimization (Enzmann and Wronski, 2019). In leukemia drug discovery, CRISPR proves useful in almost all initial stages, from its use in genetic screening in order to identify additional therapeutic targets related to the disease of interest, to its use in generating animal and cellular models for compound screening, hit validation, and lead identification and optimization.

2.1.1 CRISPR and the Identification Of Additional Therapeutic Targets in the Leukemia Genome

The process of drug discovery begins with the identification of a therapeutic target that is linked to the disease of interest. A therapeutic target is one which when inhibited or activated, produces a therapeutic effect in the diseased cell (Thorne, 2019). In a genetic disease such as leukemia, these targets are often genes in a cancerous cell. To determine which genes in the genome may be a potential target for leukemia therapy, a genetic screening test is carried out. In the screening test, large numbers of candidate genes are knocked out, inhibited, or activated. Any genetic changes that result in an abnormality in the functioning of the cell may be identified as potential drug targets. Deviations in the genome which may hinder or exacerbate the expression of the disease of interest are especially strong candidate targets.

As discussed above, CRISPR-Cas is a powerful gene editing tool that can induce DSBs in DNA in a direct, efficient, and accurate manner. The genetic screening process harnesses this power of CRISPR to be able to modify thousands of genes and assess their effects on a disease in a single experiment (Springer Nature, 2020). The basic principle of CRISPR-Cas screening is to knockout every gene that may be important in contributing towards the disease under study, but to knockout just one gene per cell. After the knocked-out cells are allowed to grow for a few days, next-generation sequencing (NGS) is performed upon them to determine which sequences are present, and thereby which cells survived. This elucidates which genes are essential for survival under normal conditions. Often, screening is done in order to identify which genes allow the cell to survive under specific conditions, such as situations of drug treatment. For example, in the situation that a leukemia cell line is resistant to a particular drug, the genetic screening test may be conducted in the presence of that drug, to determine which genes, when knocked out, enable the death or survival of the resistant cell line. Accordingly, a treatment specific to that gene can be developed (refer to Figure 3) (Spencer, 2019). Researchers often begin this process with a list of thousands of genes that may be involved in drug resistance and aim to narrow this down by CRISPR screening. From these genes, large lists of CRISPR targets are created, about 6-8 per gene, which have appropriate protospacer adjacent motif (PAM) sequences next to them (Spencer, 2019). A well-designed CRISPR screening experiment should also make use of 'control' populations of cells, which serve as a yardstick to measure the properties of the genetically knocked-out cells against.

Once the target and control populations have been identified, a pool of oligos

is to be created, which is used to make lentiviruses. Oligo pools are a diversity of oligonucleotides which are used to generate CRISPR sgRNA sequences. Each oligo must include DNA to code the targeting region of the CRISPR guide RNA, or the full sgRNA, including the target sequence. Each oligo must also have sites at its ends to allow for cloning into lentiviral gene-containing plasmids. From the plasmids, lentiviruses are produced as a pool containing thousands of CRISPR target sequences, one sequence per virion (Spencer, 2019). Researchers then infect each cell with one virion, containing one target DNA sequence, which results in the formation of the specified RNA sequence in the cell. Along with this, a Cas enzyme also must be expressed in the cells under study, for the CRISPR-Cas system to work. This Cas enzyme can either be delivered through a pool of lentiviruses that contain both the DNA that codes for the gRNA as well as the cas enzyme, or it can be integrated into the cells by using a cell line that stably expresses a Cas enzyme itself.

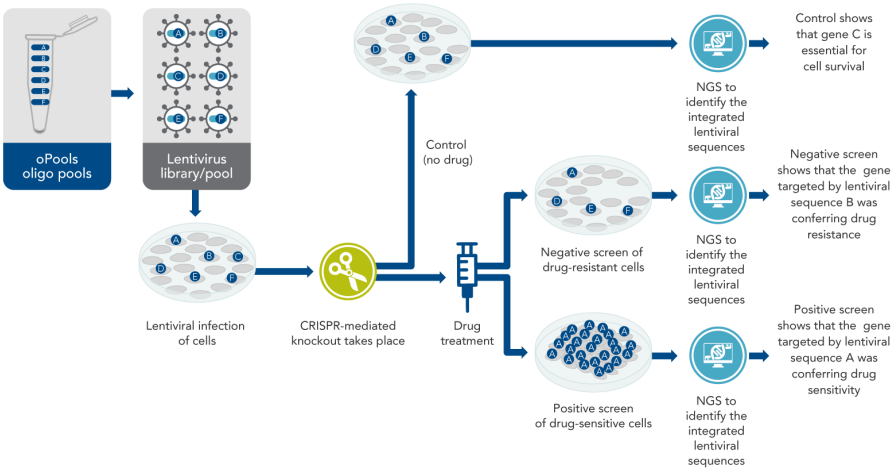

Figure 3: Figure 3: CRISPR screening using pooled oligonucleotide sequences (Spencer, 2019)

CRISPR screening can be either positive or negative. Negative screens are used to discover genes that cause drug resistance, whereas positive screens are used to look for genes that cause drug susceptibility. In negative screening, if a cancer cell line is resistant to a particular drug, the cells from that cell line will have a different gene knocked out per cell and will be treated with a drug. Some of these cells will die in response to the drug, indicating that the resistance genes in these cells were knocked out. The control for this experiment would be the cells targeted with the same lentiviral pool, but not treated with the drug. From the untreated control group, many genetic sequences will be detected since most of these cells survive, but from the experimental or test group, the genetic sequences that are not detected will be the ones that contribute to drug

resistance in cancer cells (Spencer, 2019).

In positive screening, some of the gRNAs of the screen may knockout cells that cause sensitivity to a particular drug. Most of the unedited cells, which are the ones after the CRISPR screen but before the drug treatment, will be killed by the drug. Most of the CRISPR edited cells will also be killed, since the knocked-out genes in them are not drug-sensitivity genes. Only the cells with knocked out drug- sensitivity genes will grow and will proliferate much more than any other cell type when under treatment with the drug. NGS sequencing of these cells will show the DNA that was used to code the gRNA in that cell, and from this the drug sensitivity gene can be determined (Spencer, 2019).

In summary, screens that look for the loss of sgRNAs in cancer cell lines under the treatment of a particular drug will find those gene knockouts that sensitize the cell to the drug, and screens that look for the enhancement of genes present in cells that are under treatment with the drug will find those genes which are responsible for the cell's resistance to the specified drug.

An example of genetic targets that have been identified for therapeutic use in leukemia through the employment of CRISPR-mediated screens is a study conducted by K. Tzelepis et al., in which additional therapeutic targets were identified in the AML genome. The team identified 492 AML-specific cell- essential genes, including therapeutic targets such as DOT1L, BCL2, and MEN1. The KAT2A gene was chosen as a candidate for study, and the inhibition of KAT2A resulted in the demonstration of anti-AML activity (Tzelepis et al. 2016).

Another instance of CRISPR screening that has been useful in identifying leukemia targets is the identification of genes that are essential for imatinib-induced cell death in CML, by Matthieu Lewis et al. A genome-wide CRISPR knock-out screen was used to screen for imatinib-sensitizing genes in vitro, on K562 cells. Imatinib is a tyrosine kinase inhibitor (TKI) and is often used as a form of cancer therapy. Genes that were discovered as essential for imatinib-induced cell death were proapoptotic genes such as BIM and BAX. Targeting these specific genetic sequences was hypothesized as being able to overcome drug-resistant phenotypes of CML cells (Lewis et al. 2020).

Compared to previous methods of genomic screening, such as RNA interference (RNAi), CRISPR screening has greater efficiency and specificity towards its genomic target, and thus reduces the chances of off-target effects (Springer Nature, 2020). CRISPR genetic screens also enable researchers to detect the effects of complete gene knockouts, rather than just partial reduction, which is the case in RNAi screening processes (Springer Nature, 2020). Using RNAi screening may result in the expression of certain residual low level proteins, which may be translated even after the genetic knockdown. This is because RNAi uses short pieces of RNA to inhibit protein translation, by binding to the mRNA molecule and physically preventing ribosomes from translating mRNA to proteins (Thorne, 2019). Therefore, most of the limitations faced by previous methods of genomic screening are overcome by CRISPR-Cas screening methods.

Apart from identifying genes for drug sensitivity and drug resistance, CRISPR genetic screening is also used in identifying targets for synthetic lethality. A syn-

thetic lethal interaction between genes A and B is defined as one where the loss or inhibition of just one of these genes does not significantly affect the survival of a cell, but the loss or inhibition of both these genes can prove highly toxic to the cell (Ramkumar and Kampmann, 2018). Synthetic lethality plays a large role in leukemia medicine and therapy. CRISPR can be used to identify synthetically lethal genes with the use of isogenic cell pairs - pairs of cells that are identical except for one mutation. By performing screening on these cell pairs, the sgRNA sequences that are depleted in the mutant cell line compared to the wild-type cell line would indicate synthetically lethal genes in that cancer cell line (Thorne, 2019). Recent loss-of-function genetic screens, using CRISPR, have identified new synthetic lethality leukemia targets in the genome. These include (Mair, et al., 2019): the identification of sets of AML (acute myeloid leukemia) specific essential genes (Zhao, et al., 2017) and the identification of the ENL (eleven-nineteen leukemia) protein as a specifically vulnerable target in mixed-lineage leukemia (MLL)/AF4 positive acute leukemia (Erb, et al., 2017). The main advantage of targeting synthetically lethal cancer genes for therapeutic purposes is that they are less likely to have toxic side effects, since wild-type cells are not susceptible to this therapy (Thorne, 2019).

In addition to the above methods, scientists are also exploring to exploit the use of cell senescence clinically. Senescence is a process activated by stresses, that prevents abnormal cells from proliferating, resulting in them entering irreversible growth arrest (Thorne, 2019). Using CRISPR screens and libraries, researchers can identify potential genes, which when knocked out, induce senescence in the cancer cell. Several genes are activated during cell senescence and induce senescence if excessively expressed in cancer cell lines. The discovery and exploitation of such genes may be helpful in bringing about therapy-induced senescence (TIS), which is a promising way to provide leukemia therapy (Ewald et al. 2010).

2.1.2 CRISPR and the Generation of Accurate Animal Models for Leukemia Modelling

Animal models have proven to be invaluable in the search for a better understanding of leukemia. This has led to their use ultimately aiding the treatment of patients suffering from the disease. This is also facilitated by the fact that leukemias are relatively genetically simple, since their development lies on a limited number of initiating events (Ablain, et al., 2013). Several leukemia animal models, particularly mouse models, have been used for pre-clinical purposes and have been contributory in predicting the response of patients to certain therapies, as well as in drug research and development. A pioneering instance depicting the importance of animal modelling in leukemia is that of the discovery of the leukemic stem cell by Dr John Dick in the 1990s (Cook and Pardee, 2012).

In the drug development process, animal models are of great use in the evaluation of novel therapeutic compounds and treatments. One of the biggest challenges in cancer research worldwide is the creation of robust pre-clinical

animal models which can accurately reflect a human response to a newly developed drug or therapy. This is particularly a challenge because often therapies that elicit a response in animal models do not have a significant therapeutic effect on human cancer patients (ScienceDaily, 2017). Taking into consideration the enormous cost of clinical trials, researchers are constantly on the lookout for technology that can contribute towards developing clinical models that can accurately reflect a human disease's genetics, and help reliably predict which drug possesses maximum potential to treat patients suffering from that disease, which is where the role of CRISPR comes in.

Leukemia animal models for preclinical research can be classified intro three broad types (Ablain, et al., 2013): (1) Xenografts, which refer to the transplant of tissue from one organism to another in order to model a disease in an animal, (2) Retroviral transductions, which use retroviruses to deliver nucleic acids into animal cells for modelling of a disease, and (3) Transgenic models, which refer to animal models that have had their genomes altered in certain ways to induce a specific disease in the animal. CRISPR plays an important part in the alteration of the genomes of animal models to induce genetic diseases in them, one of the diseases being leukemia.

In a study conducted by a team led by Zuzana Tothova, the team described an approach that uses CRISPR to create leukemia animal models (ScienceDaily, 2017). Using CRISPR-Cas9 to edit human hematopoietic stem cells, followed by their transplantation in mice, customized mouse models were designed to model the progression of leukemia. First, large-scale sequencing data was examined by the team, to determine which combinations of genetic mutations occur most frequently in MDS (myelodysplastic syndrome) and AML (acute myeloid leukemia). MDS is classified as a pre-malignant stage that often results in the patient developing leukemia, whereas AML is one of the most common forms of leukemia. This resulted in the discovery of nine genes which are commonly mutated in both diseases. To study these mutations in combination, the team devised a way to introduce these mutations into human hematopoietic stem cells, where leukemias originate from. Primary hematopoietic stem cells were taken from healthy donors and CRISPR-Cas9 was used to engineer them with several different combinations of the nine mutations. The general principle of CRISPR- Cas gene editing applies to the development of mouse models too (Mou et al., 2020).

The combination of mutations that did not kill the cell, and that led to an expansion of the cells over time, were the same combinations observed in actual human tumor samples. This reflected the high level of accuracy that the mouse models were capable of exhibiting. From there, these stem cells were injected into the mice's circulatory systems, where a fraction of them incorporated themselves into the bone marrow. The team then monitored the progression of leukemia in these mice. They extracted and sequenced the human cells five months later to determine which genetically modified cell lines successfully propagated and which of these mutations became more prominent over time. This was studied in both the pre-malignant and early malignant stages (ScienceDaily, 2017).

Based on data from previous studies, mutations in the TET2 gene predict the success of treatment with the drug azacitidine, while mutations in the ASXL1 gene predict resistance of the tumor cells to treatment with azacitidine. When the researchers tested the mice with azacitidine, it was found that the response in the engineered cells in the mice greatly replicated the previously studied response in human cells. It was also discovered that mutations in the SMC3 gene increased susceptibility to azacitidine, a finding that could prove useful to clinicians and patients worldwide (ScienceDaily, 2017. In this study, the efficiency and accuracy of the CRISPR-Cas system was instrumental in achieving the final result: a mouse model that could accurately represent human leukemias and interact with drugs and display responses identical to what human leukemia cells would display in a patient.

Another instance where hematopoietic stem cells were edited using CRISPR-Cas9 systems is the generation of mouse models with myeloid malignancies, by Dirk Heckl et al (Heckl et al., 2014). By delivering small combinations of sgRNAs and Cas9 using lentiviral vectors, up to five genes were modified in a single mouse hematopoietic stem cell, which led to clonal outgrowth and resulted in a myeloid malignancy. Multiple mutations were needed in a single cell since most myeloid malignancies are caused by multiple genetic alterations (minimum 3-4 in AML). Based on this, models of acute myeloid leukemia (AML) were generated, by cooperating mutations in epigenetic modifiers, transcription factors, and mediators of cytokine signaling, which recreated the mutations observed in human AML cells.

2.1.3 CRISPR and the Generation of Accurate Cellular Models for Drug Development

Another kind of modelling which is essential in leukemia research is cellular modelling. Cellular models, or cell lines, are widely used in different fields of medical research, and are particularly useful in cancer research and drug discovery. They are monoclonal populations of cells that can undergo infinite proliferation in culture, without undergoing cell senescence. These cell lines are an unlimited supply of homogenous cells for genetic and biochemical studies and can therefore be used to study genetic diseases like leukemia in detail. Animal cell lines, such as rodent cell lines, are relatively easy to generate, whereas the generation of human cell lines for cancers is particularly difficult. Despite the limitations, over a thousand known hematopoietic cell lines have been generated since the 1960s, using cells from the bone marrow, peripheral blood, or pleural effusions (excess fluid between the layers of the pleura of the lungs) of leukemia patients. While regular hematopoietic cell lines can survive in vitro for only a few days or weeks, these cell lines are able to proliferate continuously in culture while preserving most of their genetic characteristics (Kennedy and Barabé, 2008).

Human hematopoietic cell lines have been useful in the identification of leukemia-associated oncogenes, through the process of genetic screening, which has been discussed above. Cell lines have also been used in the discovery and

testing of therapeutics, from conventional chemotherapeutics to modern targeted therapies like kinase inhibitors. An example of this is the discovery that L-asparaginase could inhibit the in-vitro growth of human B-ALL (where an excessively large number of B-cell lymphoblasts are found in the bone marrow and blood) cell lines (Lazarus, et al., 1969). Cell lines have also provided validation of targeted molecular therapies such as imatinib, which was found to inhibit the growth of cell lines expressing the BCR-ABL gene, which is found in almost all patients suffering from chronic myeloid leukemia (CML) (Carroll, et al., 1997).

In the past few years, the emergence of CRISPR-Cas as a genome editing tool has accelerated the accuracy and ease with which researchers can now create cellular models to study diseases. Though animal models prove useful in the study for cancers like leukemia, they are not always an exact replica of the disease as it progresses in human cells (Cook and Pardee, 2012). Modelling the disease in derived human stem cells, in culture, enables the creation of cellular models which are almost exact representations of the disease as it occurs in the human body. This enables scientists to carefully monitors stages of leukemia development, study genetic and environmental factors that contribute to leukemia, and accurately test for the effects of drugs on leukemic cells. Genetic sequences that may be associated with leukemia can be determined by using CRISPR screening methods. Cellular models containing alterations in these genetic sequences can be generated. Using these models, the progression of leukemia can be studied, as the cell acquires subsequent mutations that may contribute to leukemia development. The interaction of therapeutic drugs at different leukemic stages may also be a subject that can be investigated using cellular models. While most genetically edited human cell lines are either used for CRSIPR-Cas screening or are transplanted in animals, some are used in isolation to model and study leukemia as well.

In a study conducted by Miguel Quijada-Álamo et al., CRISPR-Cas9 technology was used to generate stable isogenic CLL cell lines, harboring del(11q) and/or ATM mutations (Quijada-Álamo et al. 2020). Del(11q) refers to a genetic abnormality in which there is a deletion of genetic material on the long arm (q) of chromosome 11 (Ma et al., 2002). The ATM gene is one that codes for a protein which coordinates DNA repair by activating enzymes that are capable of fixing broken DNA strands (National Library of Medicine (US), Genetics Home Reference, 2020). The loss of ATM by del(11q) and gene mutation led to an increased genomic instability and hypersensitivity of the cell line to the PARP inhibitor Olaparib, in vitro, in vivo, and ex vivo. PARP inhibitors are a group of pharmacological inhibitors that inhibit the activity of the poly ADP ribose polymerase enzyme (PARP) and are used in the treatment of heritable cancers like leukemia (Chen, 2011). Additionally, it was also shown in the study that ibrutinib synergizes with PARP inhibition, which triggers synthetic lethality and improves the effects of BCR (B-cell receptor) inhibition in del(11q) cell lines and primary CLL cells. This study suggested that the use of CRISPR-Cas- generated cell models can prove to be a powerful tool to study the effects of individual and combined CLL genome alterations on the response of cells to drugs and therapies (Quijada-Álamo et al. 2020).

As discussed in the above paragraphs, CRISPR-Cas gene editing systems, although relatively in a stage of infancy, are extremely useful as tools in the research and understanding of leukemias. While these processes come with their own limitations, many of which are discussed at the end of this review, they also help advance leukemia research and drug development to a standard that has been previously unachievable.

2.2 CRISPR in Leukemia Therapy

Leukemia therapies for a patient depend on multiple factors, from the type of leukemia a patient is suffering from, to their age and overall health, as well as whether the leukemia has metastasized to other parts of a patient's body. At present, leukemia therapies are of five major types (Cleveland Clinic, 2020): (1) Chemotherapies, which are the most common forms of therapy, and are carried out by the administration of therapeutic chemicals (medications) to the patient; (2) Radiation therapy, which uses strong beams of radiation to kill leukemia cells and prevent them from proliferating; (3) Immunotherapy, which uses drugs to boost a patient's own immune defense mechanism, and includes interferons, interleukins, and CAR-T cell therapy; (4) Targeted therapies, which focus on features specific to leukemia cells in the body and are less likely to harm normal cells; (5) Hematopoietic cell transplant, where cancerous cells are killed by chemotherapy or radiation therapy, and are then replaced by new, healthy hematopoietic cells. This section of the review elaborates on how CRISPR can be used to aid two kinds of therapies for leukemia: Immunotherapy, and gene therapy for autologous transplantation. In leukemia immunotherapy, CRISPR-Cas systems are especially employed for their use in chimeric antigen receptor-T cell (CAR-T cell) therapy, which will be elaborated upon significantly in the following paragraphs. In leukemia gene therapy, CRISPR-Cas systems can be used to edit hematopoietic stem cells belonging to the leukemia patient to correct genetic mutations, and these corrected cells can then be autologously transplanted back into the patient's body. This mechanism will also be elaborated upon in the following paragraphs. Although gene editing for autologous transplantation is presently not as prevalent as CAR-T cell therapy for leukemia, it holds immense potential that can be harnessed in the future. This section aims to propose a mechanism with which CRISPR-Cas can be employed to correct genetic mutations in leukemic HSCs, which can be autologously transplanted into the patient's body for therapy.

2.2.1 CRISPR in Leukemia Immunotherapy

Immunotherapy is a form of leukemia treatment that aims to prompt the patient's own immune system to identify and destroy leukemic cells (Moffitt Cancer Centre, 2018). Under normal circumstances, the immune system protects the body from viruses, bacteria, allergens, and other foreign bodies. By enhancing the immune system to also attack cancerous cells, immunotherapy can work as a therapy for leukemia and other hematological malignancies. There

are several kinds of immunotherapy that can be used to treat leukemia (Moffitt Cancer Centre, 2018), which include allogenic bone marrow transplant, therapeutic cancer vaccines, T-cell therapies, monoclonal antibody therapies, and donor lymphocyte infusions. Gene editing has been used in the development of T-cell therapies, and CRISPR- Cas is the most efficient form of gene editing that has been applied for use in advancing CAR T-cell therapy. Adoptive T-cell immunotherapies have revolutionized cancer treatment, especially after the FDA approved Kymriah and Yes carta (CD-19 directed CAR T-cells in B-cell leukemia and lymphoma) (Zheng, Kros, and Li, 2018).

Chimeric Antigen Receptor (CAR) T-Cell Therapy:

Human lymphocytes are of three kinds: (1) B cells, which make antibodies to fight infections; (2) T-cells, which help B-cells produce antibodies and directly kill infected cells themselves; (3) Natural killer (NK) cells, which attack infected cells and eliminate viruses (Alam and Gorska, 2003). In present-day CAR T-cell therapy, T-lymphocytes are collected from a patient's body and engineered in a laboratory using gene editing mechanisms, to form chimeric antigen receptor cells. Chimeric antigen receptors (CARs) are proteins found on the surface of the engineered T-cells that allow the T-cells to recognize an antigen on the surface of target tumor cells. These CAR T-cells are then multiplied and cryopreserved, after which they are sent to the hospital where they are thawed and infused back into the patient. CAR T- cells may eradicate all cancer cells and remain in the body months after the infusion has been completed (Leukemia and Lymphoma Society, 2020) (refer to Figure 4). CAR-T cell therapies have shown very high remission rates, of up to 94 percent remission in some cases of severe blood cancer (Fernández, 2019). This is particularly an achievement, considering that CAR T-cell therapy is only prescribed to patients who have been unable to respond to other forms of clinical leukemia treatments.

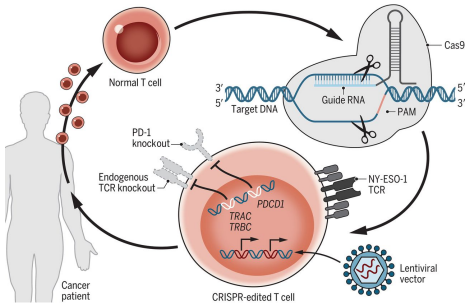

Figure 4: Figure 4: CRISPR-mediated CAR T-cell therapy (Stadtmauer et al. 2020)

Major histocompatibility complexes (MHCs) play key roles in the surveillance of aberrant proteins on the surface of tumor cells. T-cell receptors (TCRs) on the surface of T-lymphocytes recognize these aberrant proteins in combination with MHCs. However, in cancer patients, tumor cells can escape recogni-

tion by T-cells through mechanisms such as the down regulation of MHCs, or mutations. The absence of usual amounts of MHCs on the surface of tumor cells limits TCR recognition, and therefore T-cells fail to recognize and destroy tumor cells (Liu, et al. 2019). However, CAR T-cell therapy uses genetically edited T-cells, which express CARs on their surface. These CARs allow T-cells to be activated regardless of the presence of MHCs on the surface of tumor cells. A CAR consists of a binding domain (single-chain antibody fragment, ScFv), a transmembrane domain, and an intracellular signaling domain, which is responsible for the activation of T-cells. T-cells are genetically modified to express CARs on their surface, and these CARs are responsible for recognizing the tumor-associated antigen (TAA) on the surface of tumor cells. At present, the most successful CAR T-cell therapy involves targeting CD19, an antigen expressed by B-cells and B-cell malignancies. CAR T-cells have provided great advances in the treatment of hematological malignancies, with anti-CD19 CAR T-cell therapy exhibiting a 90 percent complete response rate in ALL patients (Maude, et al. 2020).

Currently, most CAR T-cell therapies use autologous T-cells, which may cause the therapy to be hampered by the unavailability of large quantities of autologous T-cells in certain leukemia patients, especially those that are either very young, or elderly. In some cases, rapid progression of the disease also results in the death of the patient before a substantial number of T-cells can be engineered and delivered to the patient (Li, Mei, and Hu, 2020). To overcome these hurdles, researchers are looking to develop "off-the-shelf" CAR T-cells from healthy donors, which can be administered to patients without the need for harvesting large numbers of autologous T-cells, and without having to keep the patient waiting until enough T-cells are genetically edited. Off-the-shelf allogenic CAR T- cells also allow for the standardization of CAR T-cell products, time for multiple cellular modifications, for the administration of multiple doses or combinations of doses of CAR T-cells for different target tumors, and for decreased cost due to industrialization of the production of CAR T-cells (Depil et al. 2020).

The major concerns in universal off-the-shelf CAR T-cell production are graft-versus-host disease (GVHD), and rejection of the infused allogenic T-cells by the host's immune system. To overcome the possibility of GVHD, the T-cell receptor (TCR) on allogenic T-cells needs to be eliminated, and to overcome the possibility of immune rejection, human leukocyte antigens class 1 (HLA-1s) need to be eliminated from the donor T-cells. Previous studies have shown that a mutation in the TCR subunit constant (TRAC) gene leads to the loss of the T-cell receptor on the T-cell's surface. Another study has shown that the beta-2 microglobulin (B2M) gene is essential for the expression of HLA-1s on the surface of the T-cells, and that a mutation in the B2M gene may result in the elimination of HLA-1s from T-cell surfaces (Liu, et al. 2016). Therefore, by targeting the TRAC and B2M genes, allogenic T-cells can be made safe for their use in the development of off-the-shelf CAR T-cells for leukemia immunotherapy. Furthermore, blocking signaling of the cell-surface molecule, PD-1 (programmed death-1), in the engineered T-cells can reverse immunosup-

pression (Riley, 2009). To make allogenic stem cell transplants a reliable therapy for leukemia, CRISPR/Cas-mediated gene editing can be employed.

To generate universal off-the-shelf CAR T-cells as mentioned above, multiple genes need to be edited simultaneously in the genome of the T-cells. The process of gene editing has to be carried out twice - once to edit the allogenic cells to disable the receptors (and thus eliminate the possibility of immune rejection) by causing mutations in the TRAC, B2M and PD1 genes, and the second time to edit the allogenic cells in order to enable them to express CARs on their surface. In a study conducted by Xiaojuan Liu et al., CRISPR-Cas9 gene editing systems were used to generate CAR T-cells with either two (TRAC and B2M) mutations (double knock-out, or DKO), or three (TRAC, B2M and PD-1) mutations (triple knock-out, or TKO), and their anti-tumor functions were tested in vitro and in vivo (Liu, et al. 2019). The T-cells had been isolated from umbilical cord blood. The efficiency of gene editing after inducing the double and triple knockouts was also tested using various methods, to check for unwanted mutations and off-target effects.

The T-cells were then subject to CRISPR-Cas9 mediated gene editing to enable them to express CARs on their surface. Anti-CD19 CAR T-cells were used in the gene editing experiments since they have been successful in treating CD19+ B-cell leukemia and lymphoma. By transducing T-cells with anti-CD19 CARs 3 days after T-cell activation, and then performing CRISPR-Cas9 electroporation on day 5, followed by the harvesting of the cells on day 15, CAR T-cells were generated. Using this protocol, CD3+ T-cells from five different donors were genetically edited to form CAR T-cells. The gene editing of these cells also showed negative effects on their proliferation, although they were able to proliferate considerably. It was also determined that the DKO CAR T-cells lost TCR and HLA-1 expression on their surface, as anticipated (Liu, et al. 2019).

Next, the cytotoxic mechanism of the CAR T-cells were evaluated in vitro. The cytotoxic mechanism of the DKO and TKO cells was shown to be similar to that of the unmodified CAR T-cells. It was also shown that multiplex gene edited CAR T-cells display anti-tumor activity equivalent and sometimes even superior to regular CAR T-cells. When evaluated in vivo in mouse models too, the mechanism of action of multiplex gene-edited CAR T-cells were not compromised. Through this study, it was concluded that CRISPR-Cas mediated multiplex genome editing is a tool that can readily be applied to the development of CAR T-cells, and through its use, allogenic off-the-shelf CAR T-cell therapy may be further investigated and may become a future possibility (Liu, et al. 2019).

2.2.2 CRISPR in Leukemia Gene Therapy for Autologous Transplantation

Another possible method in which CRISPR can be employed to treat leukemia is in the genetic editing of a patient's hematopoietic stem cells for autologous transplantation. Although this method is not widely prevalent in leukemia

therapy, it is already used for the treatment of other genetic hematopoietic disorders such as thalassemia and sickle cell disease. Hematopoietic stem cells are obtained from the patient, genetically edited to correct the prevalent mutation, and are then re- transplanted back into the patient, where they engraft themselves into the bone marrow (Morgan et al. 2017). The blood cells produced by these edited HSCs are then normal blood cells. If this method can be employed to genetically correct the mutations present in leukemic HSCs, it can be developed as a potential therapy for leukemia too.

The use of allogenic transplantation of hematopoietic stem cells (HSCs) for the treatment of leukemias is limited by the availability of suitable donors and the possibility of immunological complications. Gene therapy using autologous HSCs is a method that has the potential to overcome these limitations. The use of autologous HSCs overcomes the possibilities of graft rejection and graft versus host disease (GVHD), both of which are serious complications that may arise in allogenic transplantation between donors and recipients with reduced human leukocyte antigen matching (HLA matching) (Complications or Side Effects of Allogeneic Stem Cell Transplant - Virginia Cancer Institute, 2020). Allogenic transplantations also require suppression of the recipient's immune system immediately after the transplant has taken place. Patients also usually need blood and platelet transfusions to overcome the anemia and thrombocytopenia as a result of bone marrow suppression (Complications or Side Effects of Allogeneic Stem Cell Transplant - Virginia Cancer Institute, 2020). Therefore, autologous HSC transplants, as compared to allogenic HSC transplants, may be a preferable form of therapy for patients suffering from various forms of leukemia. CRISPR-mediated gene editing could serve as a powerful tool in the engineering of modified autologous HSCs for transplantation.

Current approaches to autologous transplantation using lentiviral vectors have exhibited clinical benefits similar to those resulting from allogenic transplants, with reduced risk of infections and complications (Morgan et al. 2017). Developments like CRISPR-Cas systems have led to investigations towards the application gene editing in HSCs. Since HSCs are self-sustaining, multipotent, and possess the ability to proliferate extensively (Hematopoietic Stem Cells — stemcells.nih.gov 2020), gene correction in HSCs should lead to persistent gene correction in the resulting cell lines. The hematopoietic system is an ideal target for gene therapy because of the ease with which HSCs can be obtained from and re- administered as intravenous infusions to the patient (Morgan et al. 2017).

HSC harvest aphaeresis is the main method for obtaining cells that can be used in HSC transplant for leukemia. Previously, HSCs were collected from the bone marrow by placing a needle into the interior of the bone to obtain these cells, in a procedure known as bone marrow aspiration. At present about 90 percent of these cells can be collected from the blood stream itself, by HSC harvest aphaeresis. This saves the pain and complexity of carrying out bone marrow aspiration (UC San Diego Health, 2020). Hematopoietic growth factors also may be administered to patients before the aphaeresis procedure is carried out (Morgan et al. 2017). Cells isolated from the blood which are CD34+ represent cells are either HSCs or HSPCs, and can be genetically modified for

transplantation (Pei, 1999). Once a suitable quantity of HSCs is derived from the patient, they can be frozen for storage and genetic modification.

In the genetic editing of hematopoietic stem cells, targeted gene editing using site-specific endonucleases such as CRISPR can be used to induce double strand breaks in the HSC genome, near the site of the leukemia-causing mutation. The cellular DNA repair mechanism will then attempt to repair the DSB using either NHEJ or homologous recombination, as discussed previously. NHEJ can be used to achieve gene disruption and homologous recombination can be used to stimulate the resynthesis of a portion of the gene using a donor strand of DNA as a template. Genes that contribute to the development of leukemia and drug resistance may be knocked out using NHEJ, whereas genes that lead to the suppression of leukemia and increase the susceptibility of leukemic cells to drugs, can be knocked-in via homologous recombination. While homologous recombination is more precise, the correction of HSCs using homologous recombination is less efficient, and further investigation must be carried out in this direction, to increase the efficiency with which homologous recombination can be used in gene editing (Morgan et al. 2017), (Liu et al. 2019). To induce DSBs, other endonucleases like ZFNs and TALENS can also be employed, but the obvious advantages of CRISPR-Cas over alternative methods is its efficiency, and flexibility of developing gRNAs for various target sites.

The genetic modifications which are produced in the HSCs must be permanent since they must be passed down to successive generations of the cell line for a therapeutic effect to occur in the patient. After modification, the genetically edited cells can be made to undergo testing to assess the purity, identity, safety, and potency of the cells, as is done in the case of other genetic hematological disorders. Before the edited cells can be transplanted back into the patient, the patient must undergo chemotherapy or radiation therapy to kill the leukemic cells and to "make space" for the engraftment of the newly edited hematopoietic stem cells. An inadequate depletion in the number of diseased hematopoietic stem cells may result in poor or incomplete engraftment of the corrected HSCs into the bone marrow. The genetically modified cells can then be infused directly into the patient or can be cryopreserved to be delivered via transfusion at a later stage (Morgan et al. 2017).

3 Limitations, Improvements, and Safety/Ethical Concerns Associated with the Use of CRISPR in Leukemia Research and Therapy

3.1 Limitations and Possible Improvements

The most significant limitation faced by researchers who choose to employ CRISPR-Cas systems for gene editing purposes, is that of off-target effects, or OTEs, which have been observed at a frequency greater that 50 percent (Uddin, Rudin, and Sen, 2020). The target specificity of the CRISPR-Cas complex

is controlled by a gRNA molecule. However, off-target cleavage activity can still occur if there is a 3-5 base pair mismatch between the gRNA and the target DNA (Omodamilola and Ibrahim, 2020). In the case of animal and cellular models, off target effects can be a particular disadvantage. This is because if there is an off-target effect in a model that has responded well to a therapeutic drug, the therapeutic response may not necessarily be due to the intended mutation, but could also be due to the off-target mutation. This would result in the animal model being largely inaccurate, and would thus defeat its main purpose entirely. To overcome this, current attempts have been made to use engineered Cas9 variants and to optimize gRNA designs. An example of this is the development of the Cas9-nickase (Satomura et al. 2017), which induces a single-strand break rather than a DSB, and can reduce the probability of off-target effects. However, Cas9-nickases are not as efficient as Cas9 nucleases. Another instance is the engineered SpCas9-HF1, which is a high-fidelity variant of Cas9 that possesses no detectable off-target activity during the gene editing process (Kleinstiver et al. 2016). In recent studies, CRISPR-Cas gene editing systems have also been shown to cause deletions in the genome which are hundreds of base pairs long and are potentially problematic mutations that previously went undetected. Such alterations in the genome could hinder the application of CRISPR as a therapeutic tool in almost any disease, not just in leukemia (Ledford , 2018).

Another limitation of the CRISPR-Cas gene editing system is the need for a PAM sequence near the site where the DSB must be induced. The necessity for a PAM sequence greatly reduces the number of potential target sites at which DSBs can be induced and gene editing can be carried out. To overcome this, multiple methods have been devised, like using RNA-targeting Cas9 variants that broaden the genetic target spectrum by mitigating PAM requirements (Strutt et al., 2018).

CRISPR-induced DSBs have also been shown to kill the cells in which they are sometimes induced and have even resulted in the target cell's growth arrest. Studies have also shown that CRISPR genome editing is less likely to result in the death of cells that have defective copies of the p53 gene, which is a vital tumor suppressor gene and prevents the onset of cancer. By resulting in the death of healthy cells with functioning p53 genes and by increasing the number of cells that contain defective copies of p53 genes, CRISPR genome editing may be raising the risks of cancer developing in cells that are edited using it (Ihry et al. 2018).

Another cause of worry concerning gene editing using CRISPR in humans for therapeutic purposes is the possibility of immunogenicity. The presence of antibodies against Cas9 is common in infants and adults. The presence of T-lymphocytes against Cas9 derived from Staphylococcus aureus is also an obstacle to therapeutic CRISPR-mediated gene editing. An immune response against the Cas9 proteins could compromise the efficiency with which therapeutic effects are elicited in patients. A solution to this problem of immunogenicity is the use of nucleases which have not yet been exposed to the human immune system, or nucleases that do not activate an immune response. An alternative

to this problem would also be to identify antigenic regions on CRISPR-Cas9 to enable deimmunization and epitope masking, or employ immunosuppression by using drugs and regulatory T-cells (González-Romero et al., 2019).

Another obstacle in leukemia therapy is the possibility of hazardous effects of CAR T-cell therapy, and thus the use of CRISPR in it. Although CAR T-cell therapy has high remission rates and shows promising effects, it has, like any other therapy, its own set of flaws and side effects. It has been linked to severe and sometimes even lethal side effects, such as neurotoxicity and cytokine release syndrome. In 2016 certain companies also reported multiple deaths in the later stages of their CAR T-cell therapy clinical trials (Fernández, 2019). The deaths reported were in trials testing CAR T-cell therapies against the CD19 antigen found in B-cells, which is the most studied target in this domain of leukemia therapy. This made the scientific community realize that the technology is not as perfect as was initially anticipated. To overcome these adverse side effects, developers are already looking to come up with the next generation of safer CAR T-cell therapies. One of these developers is Cellicitis, which has developed a form of CAR T-cell therapy that enables the engineered T-cells to act only in the presence of a specific drug (Fernández, 2019).

3.1.1 Safety and Ethical Concerns

With the emergence of a powerful tool such as CRISPR, targeted gene editing is no longer a hypothesized abstract, but an everyday reality. Gene editing instances that have violated safety and ethical protocols have led to a rise in the concern associated with gene editing. An example of this is the uproar caused by CRISPR-based gene editing of twins in China, by He Jiankui, a biophysicist who was claimed to have edited the embryos of the twins to enable them to be HIV resistant (Regalado, 2019). Another instance of possible concern is the recent statement given by Russian biologist Denis Rebrikov. In his statement, given to the New Scientist, he declared that he still aims to use CRISPR genome editing to prevent children from inheriting deafness, despite international reports stating that CRISPR isn't safe enough to use in humans yet (Page, 2020).

While the clinical applications of using CRISPR in somatic cells, to treat hematopoietic malignancies, is usually accepted widely, some concerns have been raised regarding the use of CRISPR to edit human embryos and germline cells, and in some cases, even stem cells. There is currently a moratorium imposed in most nations, regarding the use of CRISPR to edit embryos and germ cells because any mutations introduced in embryonic and germ line cells would be sure to pass on to future generations (Lander et al. 2019). The implications of doing so are not yet known and may be as harmful as causing an imbalance in the global human population in the future. There is also speculation regarding the use of CRISPR to edit stem cells, since those mutations will also pass onto successive generations of cells that arise from the stem cells. Shall these mutations turn out to be harmful, they will have passed onto whole new generations of cells inside the human body, and it would become difficult to mitigate the effect of such a complication.

4 Conclusion

CRISPR-Cas systems are a revolutionary set of technologies that have widespread applications in fields across genetics, medicine, molecular biology, and biochemistry. They surpass the potential possessed by previous tools that played similar roles in research and therapy, such as ZFNs, TALENS, and RNAi. CRISPR has a remarkable potential to be applied in the study of almost any disease or phenomenon concerning genetics and has an especially wide scope for application in studying and treating leukemia. Whether is it in genetic screens to identify therapeutic targets such as DOT1L, BCL2, and MEN1 in the leukemia genome, or in CAR T-cell therapy to engineer T-lymphocytes to achieve 94 percent leukemia remission rates, CRISPR is a tool that can be employed to further leukemia research and therapy. To advance research in the field of hematological malignancies, CRISPR-Cas is applied to genetic screens to identify therapeutic targets. It is also applied to animal and cellular models to study and model leukemia, as well as to test leukemia therapeutics on. In the clinical setting, CRISPR-Cas systems are applied in the development of new and improved immunotherapies, specifically CAR T-cell therapies, which have shown promising results as forms of leukemia therapy. It is also applied in the gene editing of HSCs for autologous transplantation, although this method is not a prevalent form of leukemia treatment at present. Despite the promising results shown by CRISPR in the progress achieved in leukemia research and therapy, there are multiple limitations that need to be addressed to make CRISPR a more efficient, accurate and safe tool. While further research must be done to address these challenges, the scientific community has come a long way since the discovery and first application of CRISPR. Considering the pace at which new discoveries are taking place in the field, within the coming years, the technology shall have certainly progressed to an extent where any fathomable genetic task can be carried out using CRISPR.

References

[11q20] Chromosome 11q deletion. *Genetic and Rare Diseases Information Center*, 2020.

[Abl13] Julien Ablain. How animal models of leukemias have already benefited patients. *Molecular Oncology*, 2013.

[Acu20] Acute leukemias and their diagnosis, acute myelocytic and acute lymphocytic leukemia. *Labpedia.net*, 2020.

[All20] Complications or side effects of allogeneic stem cell transplant. *Virginia Cancer Institute*, 2020.

[Aph] Stem Cell Harvest Apheresis. Uc san diego health. Available at:.

[ASH20] Leukemia. *American Society of Hematology*, 2020.

[ATM20] Atm gene. *National Library of Medicine (US), Genetics Home Reference*, 2020.

[BE19] A. Wronski B. Enzmann. How crispr is accelerating drug discovery. *Genetic Engineering and Biotechnology News*, 2019.

[Bra97] SE Braun. Gene therapy strategies for leukemia. *Molecular Medicine Today*, 1997.

[CAR20a] Chimeric antigen receptor (car) t-cell therapy. *Leukemia and Lymphoma Society*, 2020.

[Car20b] D. Carroll. Genome editing: Past, present, and future. *PubMed Central*, 2020.

[Cel14] Cell division and cancer. *Nature Education*, 2014.

[Cha03] R. Champlin. Selection of autologous or allogeneic transplantation. *BC Decker*, 2003.

[Che11] A. Chen. Parp inhibitors: its role in treatment of cancer. *Chinese Journal of Cancer*, 2011.

[CL20] Y. Hu C. Li, H. Mei. Applications and explorations of crispr/cas9 in car t-cell therapy. *Briefings in Functional Genomics*, 2020.

[Cle20] Leukemia management and treatment. *Cleveland Clinic*, 2020.

[CRI] What CRISPR? New scientist. Retrieved 12 September 2020, from.

[Cri20a] Crispr guide. *Addgene*, 2020.

[Cri20b] Crispr timeline. *Broad Institute*, 2020.

[Dat] Worldwide Cancer Data. World cancer research fund. Available at:.

[ea69] H. Lazarus et al. Nutritional requirements of human leukemic cells. *Experimental Cell Research*, 1969.

[ea97] M. Carroll et al. Cgp 57148, a tyrosine kinase inhibitor, inhibits the growth of cells expressing bcr-abl, tel-abl, and tel-pdgfr fusion proteins. *Blood*, 1997.

[ea02] S. Ma et al. Chromosome 11q deletion in myeloid malignancies. *Leukemia*, 2002.

[ea10] J. Ewald et al. Therapy-induced senescence in cancer. *Journal of the National Cancer Institute*, 2010.

[ea14] D. Heckl et al. Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using crispr-cas9 genome editing. *Nature Biotechnology*, 2014.

[ea16a] B. Kleinstiver et al. High-fidelity crispr–cas9 nucleases with no detectable genome-wide off-target effects. *Nature*, 2016.

[ea16b] X. Liu et al. Crispr-cas9-mediated multiplex gene editing in car-t cells. *Cell Research*, 2016.

[ea17a] J. Costa et al. Genome editing using engineered nucleases and their use in genomic screening. *Eli Lilly Company and the National Center for Advancing Translational Sciences*, 2017.

[ea17b] M. Erb et al. Transcription control by the enl yeats domain in acute leukemia. *Nature*, 2017.

[ea18] R. Ihry et al. p53 inhibits crispr–cas9 engineering in human pluripotent stem cells. *Nature Medicine*, 2018.

[ea19a] E. González-Romero et al. Crispr to fix bad blood: a new tool in basic and clinical hematology. *Haematologica*, 2019.

[ea19b] E. Lander et al. Adopt a moratorium on heritable genome editing. *Nature*, 2019.

[ea19c] J. Liu et al. Building potent chimeric antigen receptor t cells with crispr genome editing. *Frontiers in Immunology*, 2019.

[ea19d] M. Liu et al. Methodologies for improving hdr efficiency. *Frontiers in Genetics*, 2019.

[ea20a] A. Chandrasekaran et al. Different methods of delivering crispr/cas9 into cells. *Progress in Molecular Biology and Translational Science*, 2020.

[ea20b] C. De Masi et al. Application of crispr/cas9 to human-induced pluripotent stem cells: From gene editing to drug discovery. *BioMed Central*, 2020.

[ea20c] M. Lewis et al. A genome-scale crispr knock-out screen in chronic myeloid leukemia identifies novel drug resistance mechanisms along with intrinsic apoptosis and mapk signaling. *Cancer Medicine*, 2020.

[ea20d] S. Depil et al. Off-the-shelf' allogeneic car t cells: development and challenges. *Nature Reviews Drug Discovery*, 2020.

[Fer19] C. Fernandez. A cure for cancer? how car t-cell therapy is revolutionizing oncology. *Labiotech.eu*, 2019.

[Fer20] C. Fernandez. 7 diseases crispr technology could cure. *Labiotech.eu*, 2020.

[GC12] T. Pardee G. Cook. Animal models of leukemia: any closer to the real thing? *Cancer and Metastasis Reviews*, 2012.

[Imm18] Immunotherapy for leukemia. *Moffitt Cancer Centre*, 2018.

[Int20a] Interferon gamma - an overview. *ScienceDirect Topics*, 2020.

[Int20b] Interleukin 2 - an overview. *ScienceDirect Topics*, 2020.

[JK08] F. Barabé J. Kennedy. Investigating human leukemogenesis: from cell lines to in vivo models of human leukemia. *Leukemia*, 2008.

[Led18] H. Ledford. Crispr gene editing produces unwanted dna deletions. *Nature*, 2018.

[LLS20] Facts and statistics. *Leukemia And Lymphoma Society*, 2020.

[Mai] B. Mair. Genetic interaction networks in cancer cells". *Current Opinion in Genetics Development*, 54:64–72. Accessed 14 September 2020.

[Mau] S.L. Maude. Chimeric antigen receptor t cells for sustained remissions in leukemia — nejm. Available at:.

[Me D. Meštrović. How does crispr compare to other gene-editing techniques? News-Medical.net. Available at:.

[MKA+] H. Mou, Z. Kennedy, D. Anderson, H. Yin, and W. Xue. Precision cancer mouse models through genome editing with crispr-cas9. online] NCBI. Available at:.

[Mor] R. Morgan. Hematopoietic stem cell gene therapy: Progress and lessons learned". *Cell Stem Cell*, 21(5):574–590. Accessed 12 September 2020).

[NIH20] Hematopoietic stem cells. *NIH Stem Cell Information*, 2020.

[OI] O.I. Omodamilola and A.U. Ibrahim. Hilarispublisher.com. Available at:.

[Pag] M. Page. Russian biologist still aims to make crispr babies despite the risks, new scientist. Available at:.

[Pei] X. Pei. Who is hematopoietic stem cell: Cd34+ or cd34-?". *International journal of hematology*, 70(4). Available at:.

[Q M. Quijada-Álamo. Crispr/cas9-generated models uncover therapeutic vulnerabilities of del(11q) cll cells to dual bcr and parp inhibition". *Leukemia*, 34(6):1599–1612. Accessed: 19 September 2020.

[RA20] Magdalena Gorska Rafeul Alam. Lymphocytes. *Journal of Allergy and Clinical Immunology*, 2020.

[Reg] A. Regalado. China's crispr babies: Read exclusive excerpts from the unseen original research. Available at:.

[Ril] J. Riley. Pd-1 signaling in primary t cells". *Immunological Reviews*, 229(1):114–125. Accessed: 21 September 2020).

[RK] P. Ramkumar and M. Kampmann. Crispr-based genetic interaction maps inform therapeutic strategies in cancer". *Translational Cancer Research*, 7(S1):61– 67. Accessed 14 September 2020.

[Sat] A. Satomura. Precise genome-wide base editing by the crispr nickase system in yeast". *Scientific Reports*, 7(1). Accessed: 27 September 2020.

[Spe] Netanya Y. Spencer. Overview: What is crispr screening?". Integrated DNA Technologies [online] Available at:.

[Sta] E. Stadtmauer. Crispr-engineered t cells in patients with refractory cancer". *Science*, 367(6481):7365. Accessed: 22 September 2020.

[Str] S. Strutt. Rna-dependent rna targeting by crispr-cas9. Available at:.

[TF_20] Gata-1, klf-1 and ikzf1 - transcription factors in hematopoiesis. *The Human Protein Atlas*, 2020.

[Tho] Lucy Thorne. Target identification and drug discovery with crispr". *Biocompare*. Available at: (Accessed 13 September 2020).

[Tze] K. Tzelepis. A crispr dropout screen identifies genetic vulnerabilities and therapeutic targets in acute myeloid leukemia". *Cell Reports*, 17(4):1193–1205. Accessed: 19 September 2020.

[unka] The complete guide to understanding crispr sgrna, synthego. Retrieved 12 September 2020, from.

[unkb] Nci dictionary of cancer terms. Available at:.

[URS] F. Uddin, C. Rudin, and T. Sen. Crispr gene therapy: Applications, limitations, and implications for the future". *Frontiers in Oncology*, 10. Accessed: 21 September 2020).

[Vid] A. Vidyasagar. What is crispr? livescience.com Available at:.

[YI18] P. Forterre Y. Ishino, M. Krupovic. History of crispr-cas from encounter with a mysterious repeated sequence to genome editing technology. *Journal of Bacteriology*, 2018.

[Zha] D. Zhao. Synthetic essentiality of chromatin remodelling factor chd1 in pten-deficient cancer". *Nature*, 542(7642):484–488. Accessed 14 September 2020.

[ZKL] P. Zheng, J. Kros, and J. Li. Approved car t cell therapies: ice bucket challenges on glaring safety risks and long-term impacts". *Drug Discovery Today*, 23(6):1175–1182. Accessed: 21 September 2020).

[Zus17] Karen Zusi. A new crispr-engineered cancer model to test therapeutics: New technique for modeling human leukemia in mice may aid drug discovery, clinical trials. *ScienceDaily*, 2017.