Exploring the Role of CRISPR in the Treatment of Bronchogenic Carcinoma

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Abstract

CRISPR-Cas technology has emerged as a revolutionary tool in the field of genetic editing, enhancing the treatment efficacy for several genetic diseases, including cancer. Its impact and efficiency surpasses those of the previous gene editing methods such as Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), making it an invaluable tool in modern medicine and research. This review primarily focuses on the applications of CRISPR in treating lung cancer. CRISPR has been used in screens, by identifying therapeutic targets such as oncogenes and tumor suppressor genes. Additionally, CRISPR has also been utilized to induce synthetic lethality in cryo-shocked tumor cells, wherein advancements in the cell carrier systems improved the delivery efficiency of the CRISPR-Cas system. Furthermore, CRISPR's feasibility in treating lung cancer by T-cell modification was explored, and recent clinical trials yielded encouraging results, with great potential for future developments that can result in T-cell modification becoming a promising and safe treatment option for bronchogenic carcinomas. This review focuses on discussing the role of CRISPR in treating lung cancer, and additionally showcases certain limitations that hinder CRISPR's complete domination in this field, while also presenting solutions and strategies used to minimize these limitations.

1 An Introduction to CRISPR and Lung Cancer

1.1 Genome Editing

Genome editing is a method that enables the precise modification of an organism's DNA with base pair specificity, through insertions, alterations, and deletions in specific locations in the genome. This revolutionary tool has been employed in research and it currently serves to be a promising approach for treating several human diseases. Nuclease-mediated genome editing functions by utilizing enzymes known as nucleases to induce a double stranded break

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(DSB) in the DNA sequence of the specific gene. After the DSB is created, the cell uses its repair mechanisms to mend the break. The two repair systems and pathways in place are non-homologous end-joining (NHEJ) and homologous recombination (HR). During NHEJ, a series of proteins cause the broken ends to join together, sometimes inserting or deleting nucleotides at the cleavage site. During homologous recombination, polymerases use undamaged DNA to synthesize a complementary DNA strand and the DNA sequences that are homologous to the damage site are inserted (Porteus, 2016).

There are three nucleases that induce DSBs within the genome, namely, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats, along with the CRISPR associated proteins (CRISPR-Cas). Meganucleases, the first nuclease-based gene editing technology (Randhawa and Sengar, 2021), will not be discussed in this review (refer to Figure 1). Currently in the world of research, CRISPR is the newest, most dominant and efficient method of gene editing, although ZFNs and TALENs are still in use for agricultural and medical purposes (Carroll, 2017). CRISPR has surpassed ZFNs and TALENs with its editing efficacy. Combined with its cost effectiveness and ease of use, CRISPR has therefore become the more preferred genetic editing tool (Alaa A. A. Aljabali, El-Tanani and Tambuwala, 2024).

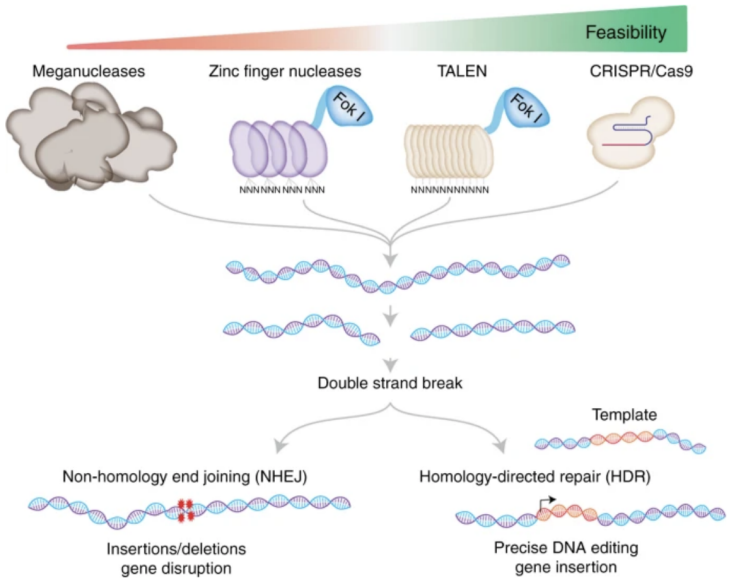

Figure 1: The evolution of gene editing technologies (Adli, 2018).

CRISPR-Cas is made up of the repeat-spacer arrays, which are transcribed into CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA), as well as Cas genes that encode Cas proteins with endonuclease activity. CRISPR

was first accidentally discovered by Ishino and his team, when they identified palindromic DNA sequences that were repeated and interrupted by spacers in the bacteria Escherichia coli. In 1990, Francisco Mojica coined the term CRISPR after finding similar sequences in other prokaryotic organisms. The function of CRISPR was later elucidated in 2007, revealing that small fragments of viral DNA, known as spacers, were inserted into the CRISPR array, which caused bacterial cells to become immunized against subsequent viral attacks (Asmamaw and Zawdie, 2021). This groundbreaking technology, an RNA-guided defense mechanism, is derived from bacterial and archaeal adaptive immunity systems. It exists within most bacteria and archaea, and inhibits phages, viruses and foreign particles from entering the cells. When attacked by these unknown particles, the CRISPR-Cas system slices the foreign DNA into small fragments which are then integrated into the CRISPR locus—a region with short repeat sequences separated by spacers. If invaded by the same particle, the CRISPR-Cas system therefore has the ability to recognize this virus, cleaving the target, and therefore effectively protecting the host organism (Xu and Li, 2020).

1.2 Mechanism of CRISPR

There are six types of CRISPR-Cas types, and several subtypes, with the most common and popular one being the type II CRISPR-Cas9, which only uses a single Cas protein (Makarova and Koonin, 2015). The Cas-9 protein is isolated from the Streptococcus pyogenes bacteria. The two principal components in the CRISPR system are the guide RNA (gRNA) and the CRISPR-associated protein 9 (Cas-9). It is an endonuclease that consists of 1368 amino acids, in charge of precisely cutting the targeted gene of interest, acting as a "genetic scissor" that causes the DNA double stranded break (refer to Figure 2). It contains two regions, the first being the recognition lobe, which has specific recognition domains that controls the binding of the guide RNA. The second region, the nuclease lobe, comprises of the RuvC domain protein, which cleaves the non-targeted DNA strand; the HNH domain, which cleaves the targeted DNA strand; and the Protospacer Adjacent Motif (PAM) interacting domains, which determines which PAM sequence is recognized and ensures specificity. The PAM is a short DNA sequence that is crucial for the system's ability to recognize and bind to its target DNA. The guide RNA is composed of the CRISPR RNA (crRNA) and the trans-activating CRISPR RNA (tracrRNA). The crRNA is around 18 to 20 base pairs long that identifies the target DNA by pairing with the sequence, and the tracrRNA consists of extended loops that acts as the binding framework for the Cas-9 nuclease (Asmamaw and Zawdie, 2021). The gRNA, in gene editing, can be artificially engineered by integrating the crRNA and tracrRNA to form a chimeric single guide RNA (sgRNA), for the purpose of targeting the desired gene of interest (Ran et al., 2013).

The mechanism of CRISPR Cas-9 can be summarized into three stages: the recognition phase, the cleavage phase, and the repair phase. In the recognition phase, the engineered sgRNA (a combination of crRNA and tracrRNA) identifies the desired sequence using its 5'crRNA complementary base pair element.

Without the presence of sgRNA, the Cas-9 protein is dormant, or inoperative. During the cleavage stage, the Cas-9 endonuclease creates a break, 3 base pairs upstream to the PAM sequence, and the protein identifies this sequence at 5'-NGG-3', where N can be any of the four nucleotides (A, G, C, or T). When the Cas-9 protein locates the target site and the suitable PAM sequence, it stimulates and brings about local DNA melting and subsequently, the emergence of an RNA-DNA hybrid. Ensuing this, the Cas-9 enzyme can finally act for inducing the DSB, as the "genetic scissors". The HNH domain, as previously mentioned, cuts the complementary strand, whereas the RuvC protein breaks the non-complementary strand, producing the DSB ends. Following this, host cell machinery carries out the repairs (Asmamaw and Zawdie, 2021). The DSB created by the Cas-9 endonuclease can be repaired by the method that is more susceptible to errors but more efficient during repair, which is nonhomologous end-joining, with random detrimental insertions and deletions of bases at the site of the cut, or it could be repaired with the more precise homology directed repair (HDR) (Jiang and Doudna, 2017).

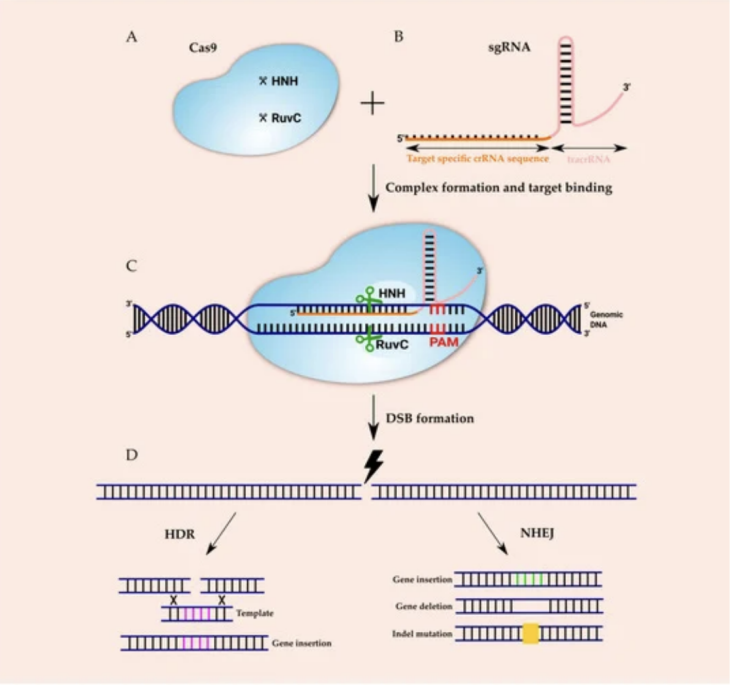

Figure 2: Diagrammatic representation of the mechanism of CRISPR Cas-9 during gene editing (Janik et al., 2020).

There are several methods for the delivery of CRISPR Cas to the desired cells, including non-viral, physical, and viral methods. Non-viral methods include cationic polymers which condense the nucleic acids into nanoparticles, pro-

tecting them while simultaneously easing the transport across the lipid bilayer (O'Keeffe Ahern et al., 2021). Physical delivery methods include microinjection technology, using needles to insert into embryos, helped by special microscopes and micro-manipulators; electroporation, which functions by disrupting the lipid bilayers with electric pulses, thereby increasing their permeability for delivery; and hydrodynamic tail-vein injection (HTVI), where the vector is dissolved in physiological saline, and injected with high pressure into the tail vein (Du et al., 2023). For in-vivo CRISPR-Cas delivery, adeno-associated viruses (AAVs) have been established as the favorable potential viral vectors. These are non-pathogenic viruses that have displayed great efficacy in delivering Cas-9 and gRNA to target cells. These viruses recognize the target cell, and enter, releasing their viral genome, which contains the genome editing nucleases. This undergoes replication and the viral particles exit the cells and infect the adjacent cells, repeating until numerous cells contain the nucleases. Additionally, lentiviral vectors contain the ability to deliver bigger payloads as compared to AAVs, making them better suited in situations for the delivery of substantially bigger DNA fragments or several genes (Alaa A. A. Aljabali, El-Tanani and Tambuwala, 2024).

1.3 Carcinomas

1.3.1 Cancer

Cancer, the world's leading cause of death, is a disease that can arise in several different parts of the body, with malignancies having the potential to develop in most tissues, including in the lungs.

Cancer is classified as a genetic disease and can be caused by errors during cell division, changes that occur in the DNA due to environmental factors such as ultraviolet rays, the ingestion of harmful chemical compounds present in tobacco or through inheritance. Mutations in the genome result in the conversion of proto-oncogenes into oncogenes. One method is by triggering the activation of certain proteins permanently, that typically change between the active and inactive state. When activated, the products release proliferation stimulating signals. On the other hand, mutations can also cause the repression of tumor suppressor genes, which are those that prevent cell division from occurring, and encourage programmed cell death. As a result, these mutations cause accelerated cell growth, leading to the formation of tumors (National Cancer Institute, 2021).

Cancer cells occur when the cells undergo uncontrollable cell proliferation. There are several differences between normal and cancerous cells (refer to Table 1).

Normal body cells	Cancerous cells
Proliferation is carefully controlled by the release of growth promoting signals that specifically instruct for the cell to undergo division.	Cancerous cells proliferate in the absence of signals that instruct them to reproduce.
Typically, when cells grow old, become damaged, they are instructed to undergo programmed cell death, also known as apoptosis.	Cancer cells ignore these signals and perpetually grow.
Normal body cells possess only a limited ability of cell division.	Cancerous cells have an unlimited potential for proliferation.

Table 1: A few main differences between normal body cells and cancerous cells (Nature Education, 2014).

1.3.2 Bronchogenic Carcinomas

Bronchogenic carcinomas, more commonly referred to as lung cancer, is a cancer that originates in the tissues lining the airways of the lungs. It is one of the most lethal forms of cancer, being the leading cause of cancer deaths, according to statistics by the World Health Organization. In 2020, 2.21 million cases were reported, with a staggering number of deaths totaling up to 1.80 million. (World Health Organization, 2022).

There are two types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Small cell lung cancer is a rapidly spreading malignant tumor that is caused primarily due to smoking. It is made up of small cells with minimal cytoplasm, indistinct and undefined cell boundaries, grain like chromatin and an unnoticeable or absent nucleoli. It displays characteristics such as growth loops, oncogene activation and inactivation of tumor suppressor genes, such as the retinoblastoma gene RB1 (Van Meerbeeck, Fennell and De Ruysscher, 2011). Small cell carcinoma is a type of SCLC, and it is also referred to as oat cell cancer. It is a highly aggressive cancer that appears like oval oat shaped cells under a microscope (National Cancer Institute, 2011b) and it is the most common type of SCLC. Combined small cell carcinoma is a rare subtype of lung cancer which has features of both SCLC and NSCLC in the same tumor (National Cancer Institute, 2020). Non-small cell lung cancer is the most common type of lung cancer, contributing towards approximately 85% of all lung cancer cases. NSCLC has three subtypes: adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Adenocarcinoma is the most common subtype of NSCLC, especially in non-smokers, and it begins in the glands that produce mucus (Myers and Wallen, 2019). Squamous cell carcinoma is present in the flat cells that line the inside of the airways in the lungs, and it often occurs in the central part of the lung near the bronchus (Sabbula and Anjum, 2021). Large cell carcinoma is a rarer subtype, making up only 9% of all NSCLC cases. It is

an aggressive form of NSCLC that can appear in any part of the lung, although it is commonly found in the outer edge, and it tends to grow and spread rapidly (Fan and Schraeder, 2011).

1.4 CRISPR and Lung Cancer

Chemotherapy is often used as a treatment method for all types of cancers, including lung cancer. However, there are severe side effects that are caused. Chemotherapy not only destroys cancer cells but causes damage to healthy body cells (National Cancer Institute, 2022). Thus, the CRISPR-Cas system proves to be a promising approach due to its high efficacy and efficiency. This literature review elucidates the role of CRISPR in treating bronchogenic carcinomas. First, this review will discuss the use of CRISPR in identifying potential target sites and CRISPR screening (specifically in EGFR mutant lung cancer) in the treatment of lung cancer, followed by the use of CRISPR in inducing synthetic lethality, delivered by cryo-shocked tumor cells, and lastly CRISPR's role in T-cell modification.

2 Using CRISPR to Treat Lung Cancer

2.1 Identification of potential therapeutic targets of lung cancer

Target identification is important in order to develop effective treatment methods. It involves finding suitable targets, which are linked to the particular disease (Ashenden, 2021). By identifying the targets, treatment methods can be developed wherein the cancer cells are targeted and the normal cells remain relatively unharmed.

As previously explained, oncogenes and tumor suppressor genes are pivotal in the study of cancer, and cancer is caused due to mutations in these key genes. When proto-oncogenes mutate, they are referred to as oncogenes, and these can then disrupt bodily processes, leading to uncontrolled, continuous cell proliferation, forming tumors. They encode proteins that propel the cell cycle and promote cell growth and are thought to be the "accelerators" as they drive cell division. Tumor suppressor genes, on the other hand, normally function to prevent cell division and induce apoptosis, or programmed cell death. Tumor suppressor genes act like "brakes" on cell division and thus prevent the formation of tumors.

CRISPR offers an efficient method to target and edit genes and has been used to target oncogenes such as EGFR, KRAS, FAK, GRM8, SMAD3, SMAD4 and MET. CRISPR has been used to knock out the EGFR gene, which results in the reduced growth of lung adenocarcinoma cells and tumors, tested in mice. Additionally, the KRAS G12S mutation, present in A549 cells has stopped their development and decreased the size of the tumor in mouse models. The FAK gene, responsible for aiding movement and evading death, when silenced, the suscep-

tibility to ionizing radiation therapy of the lung cells is increased. CRISPR was used to activate the GRM8 gene and the point mutation in the A112G single nucleotide polymorphism (SNP) established GRM8's role in promoting cell proliferation in squamous cell lung carcinoma. On the TGF-β pathway, the silencing of the SMAD3 or SMAD4 genes which act downstream, reduced Myocardin mRNA expression, impairing TGF-β-induced epithelial-mesenchymal transition and metastasis. The deletion of the 14th exon in the MET gene in NSCLC cells leads to increased cell migration, tumor spreading and metastasis, or the ability of the cancer to spread to other parts of the body. Through the HGF/MET axis, the hepatocyte growth factor associates with the MET receptor, leading to cancer growth. A mutation known as MET exon 14 skipping hinders the proper development of the MET protein, resulting in a more aggressive cancer development.

Another target is the tumor suppressor genes. Mutation in these genes is cancer causing due to continuous proliferation of cells. Using CRISPR-Cas9, researchers have discovered the functions and particular properties of tumor suppressor genes. In KRAS-driven lung adenocarcinoma, the deletion of KEAP1 using CRISPR overactivated the NRF2 pathway, increasing the speed of tumor growth. Inactivating the LKB1 gene displayed its role in tumor suppression. Similarly, deletion of PTEN or FGA by CRISPR increased NSCLC cell proliferation, migration, and metastasis in mouse models. Therefore, by knocking out or deleting tumor suppressor genes, their role in regulating and preventing tumor growth was identified (Ju et al., 2023).

2.2 Applications of CRISPR screening in lung cancer treatment

Cancer treatments often fail due to drug resistance. Drug resistance occurs when cancer cells no longer respond to a particular drug that should ideally destroy the cell (National Cancer Institute, 2011a). Certain cancer cells, or drug-tolerant persisters, endure and outlast the treatment, making the cancer drug resistant (Pfeifer et al., 2024).

CRISPR-Cas9 technology is being used for genetic screening to locate potential mechanisms of drug resistance in non-small cell lung cancer (NSCLC). Previously, RNA interference, or (RNAi)-based screens, were the main method for genome-wide loss-of-function studies, efficiently locating genes that impact tumor response to chemotherapeutic agents and studying signaling pathways. However, RNAi-based screens have several key limitations, such as off-target effects and its inability to entirely prevent gene expression, especially in genes that are abnormally overexpressed. CRISPR-Cas9 presents greater precision, as it allows for the activation or repression of gene expression, precise genome editing with lesser false positives and decreased off-target effects. As a result, CRISPR-Cas9 is now widely applied in the screening of drug resistance genes in several cancers (Huang et al., 2022).

Around 10-20% of lung adenocarcinoma tumors possess mutations in the EGFR gene. EGFR tyrosine kinase inhibitors, which are drugs like gefitinib

or osimertinib, can target these mutations, however patients who are in an advanced stage of cancer have the likelihood of gradually developing resistance towards these drugs. According to research conducted previously, several changes in the genome, including secondary mutations in the EGFR gene, oncogenic mutations and gene fusions can result in resistance. Although, recent studies suggest several non-genetic factors such as epigenetic reprogramming and the YAP/TEAD pathway (members of the Hippo signaling pathway) can lead to persistence. Therefore, targeting these early persister cells could possibly be a more efficient method in preventing drug resistance from developing.

To study EGFR mediated drug resistance, researchers used CRISPR-based screens to study genes involved in resistance to EGFR inhibitors in lung cancer. There are two types, CRISPRn screens (knockout), and CRISPRa screens (activation) to pinpoint the genes and pathways that are responsible for EGFR resistance in EGFR-caused cancer. It is found that resistance is caused by a few major pathways, with several resistance genes connected to the Hippo pathway. The Hippo pathway has a kinase cascade that controls the TEAD-dependent transcription through phosphorylation of the co-activators YAP1 and WWTR1, and inactivation of this pathway causes higher expression of these co-activator genes. These genes control several cellular processes such as proliferation, cell adhesion, and more. Treatment of EGFR mutant lung cancer cells with EGFR inhibitors leads to rapid activation of YAP1/WWTR1/ TEAD-dependent transcription, crucial for cancer cell survival. By blocking this activation, cancer cell persistence decreases. Additionally, the inhibition of YAP1/WWTR1/ TEAD-dependent transcription can restore sensitivity to Osimertinib in resistant cancer cells and prevent the development of future persistent cancer cells. Therefore, targeting the Hippo signaling pathway could be an effective strategy to combat drug resistance in lung cancer. CRISPR screens help identify resistance pathways, and targeting the Hippo/YAP1/WWTR1/TEAD axis shows promise in overcoming drug resistance, however, more in vivo and clinical studies are needed (Pfeifer et al., 2024).

2.3 Targeting Lung Cancer using Cryo-Shocked tumor cells with CRISPR Cas9

Synthetic lethality is the state at which two genes are damaged and impaired simultaneously, leading to cell death. The two genes are inactivated together, and the loss of either gene alone does not impact cell survival. (Liu et al., 2024). Tumor cells typically develop due to mutations present in oncogenes, and they become more dependent on some genes that are essential for their survival. Theoretically, tumor cells that possess the particular mutation can therefore be killed by targeting and inhibiting the other gene using synthetic lethality (since the tumor gene is mutated, and the other gene is inactivated, this uses principles of synthetic lethality). The tumor cells are therefore targeted while the normal human cells are unharmed. Synthetic lethality offers a viable solution for targeting the "undruggable" oncogenes while reducing damage to healthy tissues. The treatment, which involves cryo-shocking tumor cells to deliver CRISPR, is

a form of synthetic lethality. CRISPR can be used to induce synthetic lethality to treat lung cancer, and it revolves around a lung-targeted drug delivery system utilizing CRISPR-Cas 9 in order to treat NSCLC. This strategy primarily aims at disrupting the cyclin-dependent kinase 4 genes (CDK4) present in tumors, which is an important protein essential in the regulation of the cell cycle. By targeting CDK4 and knocking down this gene using CRISPR, a condition known as synthetic lethality is induced. As the CDK4 in cancer cells with KRAS mutations are blocked, this therapy takes advantage of the weaknesses in those tumor cells. This leads to their targeted death while leaving normal cells, which do not have those mutations, unharmed.

In this clinical trial, synthetic lethal interactions have been used. Over 25% of NSCLC involves mutations present in the Kirsten rat sarcoma virus (KRAS) oncogene. Recently, research has recognized multiple synthetic lethal interactions in KRAS-mutant NSCLC, allowing for new opportunities to improve treatment results.

The delivery of CRISPR uses both active and passive dual-targeting mechanisms in order to suppress the CDK4 genes. Active targeting increases the amount of the drug delivered to the target cell. Passive targeting, on the other hand, exploits the naturally occurring attributes in the tumors, such as increased permeability, that takes place under certain conditions. Hypoxia, or inflammation, is a particular characteristic typically present in tumors. When tumors are developing exponentially, they either use new vessels or envelop blood vessels, which leads to the formation of leaky vessels that are more permeable, allowing the drug system to accumulate in the stroma of the tumor (Attia et al., 2019). A new, novel vector was used for the delivery of the CRISPR-Cas9: cryo-shocking tumor cells. The study primarily explores the role of cryo-shocked tumors as gene targeting carriers for CDK4 genome editing, in order to induce synthetic lethality in KRAS-mutant NSCLC cells. Cryo-shocking is the process by which the cells (in this case A549 cells) are exposed to extremely low temperatures, and this rapid cooling induces stress responses in the cells which causes changes in their viability and disease-causing mechanism. The A549 cells, which are lung carcinoma epithelial cells (common KRAS-mutant NSCLC cells) are used as a vehicle for the in vivo delivery of CRISPR-Cas9. After undergoing liquid nitrogen treatment (LNT), these cells were cryo-inactivated, in order to quickly suppress its pathogenicity. Although the cell has been suppressed, it maintains its surface structure. The CD44 glycoprotein helps target the lungs by trapping CRISPR-Cas9 plasmids, which are delivered using a positively charged agent called lipofectamine 3000 to inactivate CDK4 genes. This is attached to the deactivated A549 LNT cells so it can be carried to the lung tissues (refer to Figure 3). Inside the tumor, the CRISPR-Cas9 system knocks down the CDK4 genes, leading to the death of the KRAS-mutant NSCLC cells, while leaving the normal cells functional and unharmed (Liu et al., 2024).

Although traditional delivery vectors such as viruses are known for their efficiency in regard to gene editing, there are problems associated with immunogenicity, off-target effects, and more, which restrict their potential for in vivo treatment, making cryo-shocked cells a more viable choice. The safety of the

A549 cells was also evaluated. In vivo studies utilized injections into mice: those injected with live A549 cells led to the formation of tumor nodules, whereas no indications of tumor growth was found in mice injected with the LNT cells. These findings suggest that the cryo-shocked A549 cells are safe to administer in vitro and in vivo. Therefore, cryo-shocked tumor cells are seen to be a more favorable option as compared to traditional vectors due to the lack of issues that are typically associated with them.

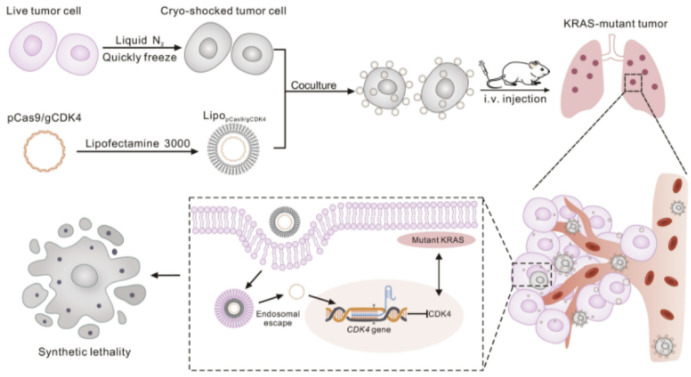

Figure 3: Process of using LNT cells containing CRISPR-Cas9 nanoparticles for treating KRAS-mutant NSCLC (Liu et al., 2024).

The study, 'Cryo-shocked tumor cells deliver CRISPR-Cas9 for lung cancer regression by synthetic lethality', investigated how CRISPR is used to induce synthetic lethality in lung cancer cells. Tumor cells often have certain mutations that make them dependent on certain genes, in order to maintain their growth, and survive. Therefore, by targeting the second gene with a synthetic lethal interaction, cancer cells can be selectively killed, without impacting the normal cells. Thus, this approach focuses on killing only the cancer cells, using a novel method of delivery of CRISPR, which was cryo-shocking tumor cells.

2.4 T- Cell Genetic Modification

T-cells, T-lymphocytes or thymocytes are a type of white blood cell that aid in protecting the body from infection and cancer, and they play an important role in the immune system. The most common types of T-cells are CD4+ T-cells, which are the helper T-cells; and the CD8+ T-cells, which are the cytotoxic, or killer T-cells (Sauls, McCausland and Taylor, 2020).

Tumor immunotherapy is a type of cancer treatment that uses the body's immune system to fight cancer, and it is a promising strategy. Tumor cells continuously avoid immune detection by switching on negative regulatory pathways, or checkpoints, and therefore, suppressing the human body's immune response. The checkpoint cytotoxic T-lymphocyte protein 4 (CTLA4) aids in controlling

the activation of T-cells by competing with the CD28 molecule. CTLA4 and CD28 both attempt to bind to the same ligand, however, when CTLA4 binds to it, the T-cells are prevented from activating completely. Another protein that acts as a checkpoint, known as programmed cell death 1 (PD-1), which is found on the surface of T-cells, binds to the programmed death ligand 1 (PD-L1) on tumor cells, and this binding acts as a barrier in fighting cancer as it results in the death of T-cells (apoptosis) and prevents the T-cells from proliferating, weakening the body's ability to fight the tumor. This in turn increases the difficulty in defeating this deadly disease. Under normal conditions when PD-1 and PD-L1 bind, the activity of T-cells is reduced to prevent unnecessary harm and autoimmunity. Tumor cells exploit this mechanism by expressing PD-L1, which prevents T-cell activation, allowing the cancerous tumors to develop and grow. Additionally, tumor-infiltrating lymphocytes (TILs), which are immune cells present in tumors, become weakened due to their exposure to cancer cells and continuous stimulation by tumor antigens, reducing their ability to fight cancer. TILs also contain high levels of PD-1, further repressing their ability to attack the tumor. By hindering the PD-1 and PD-L1 interaction, T-cell response can be enhanced, and therefore, the cancer can be fought. CRISPR-Cas9 can be used in controlling tumor immunity, particularly in editing and targeting specific genes in several pathways that are involved in the immune response. It can impact several signaling pathways that are associated with inflammation and tumor immune resistance, such as the IFN signaling pathway, the ERK/MAPK pathway, and the PI3K/AKT pathway (Xu et al., 2022).

A study was conducted in order to evaluate the efficacy of using T-cell modification with refractory NSCLC. CRISPR-Cas9 technology was used to edit the PD-1 gene present in T-cells. Two sgRNAs were chosen and along with the Cas9, they were introduced into the T-cells by using electroporation (Lu et al., 2020), a method where an electric pulse is used to introduce DNA into cells by creating temporary fenestrations in the plasma membrane to allow for gene delivery (De Vry et al., 2010). The reduction in the PD-1 expression was seen and additionally, the edited T-cells possessed more CD8+ T-cells, with better cell viability (refer to Figure 4).

According to this study, the persistence of the edited T cells present in the blood and within the tumors suggested a potential effectiveness, and it appears to be a safe form of treatment. Additionally, the side effects observed were minimal. An extremely major concern while employing CRISPR-Cas9 technology is off-target effects. Within the study, however, the targeting of PD1 displayed low mutation numbers, and the off-target effects were minimized due to the fact that this experiment was conducted ex vivo. This study displayed low mutation numbers in off-target regions, and most of the mutations were present in the non-coding areas, reducing the risk of harmful effects manifesting within the patients. Frameshift mutations (insertion or deletion of nucleotides that are not in multiples of three) and non-frameshift mutations (insertion or deletion of nucleotides in multiples of three) were minimal. However, while this worked in one patient, this study did not garner the expected response that was desired. No objective tumor responses were observed. Several potential

factors could have contributed to this outcome, namely, the insufficient T-cell expansion, the lack of antigen specificity, as well as the tumor cells not being reliant on PD-L1. Although all the patients were reported to have PD-L1+ tumors with an immunohistochemistry method, this does not necessarily equate to being dependent on PD-L1. The lack of response can also be attributed to T-cell exhaustion due to continuous exposure to tumor antigens. Additionally, the lack of viable target antigens for lung cancer, unlike other types, reduces the efficacy (Lu et al., 2020).

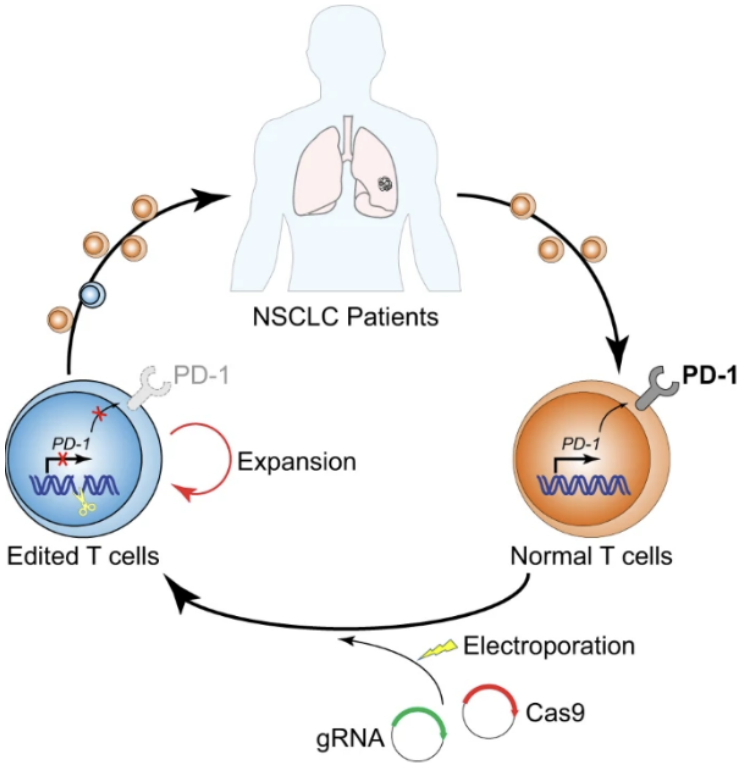

Figure 4: T-cell modification for NSCLC patients (He, 2020).

This study indicates a potential usage of T-cell modification, specifically in treating advanced lung cancer. Although, from a clinical perspective, this study was not particularly strong, this is one of the first trials to test out the efficacy of CRISPR for T-cell modification in lung cancer, and several positive outcomes were present, such as the low off-target effects, as a result, it offers a feasible strategy to allow for T-cell modification. Future trials should aim to use higher sample size, with larger quantities of tumor-reactive T-cells, and a more efficient gene-editing system. With further investigation, this treatment method has the potential to emerge as the next prominent strategy for treating lung cancer.

3 Limitations

There are several limitations that manifest while utilizing CRISPR-Cas, and in this review a few main problems are mentioned and explored. Currently, as mentioned previously, one of the biggest issues with CRISPR-Cas are the potential off-target effects that can occur, which have a tendency to occur at greater than 50% (Uddin, Rudin and Sen, 2020). However, off-target effects can be reduced, by utilizing the Cas9 nickases, in place of Cas9 nucleases, creating a single stranded break instead of a DSB. Nickases create small cuts that can be precisely repaired, inducing HR instead of NHEJ, although nickases are not as efficient as nucleases (Satomura et al., 2017). However, as seen in the study of T-cell modification to treat lung cancer, since the cells were edited ex vivo, the problems posed by off-target effects decreased as mutations were lower in number.

Another limitation of the CRISPR-Cas system is the requirement of a PAM sequence close to the site wherein the DSB is induced. Since the PAM sequence is needed, the number of potential target locations where DSBs can be created decreases. Possible solutions, such as Cas9 variants like SpCas9-NG offer a minimal PAM requirement, allowing for broader therapeutic applications (Uddin, Rudin and Sen, 2020).

Another drawback is the possibility of immunogenic toxicity. Many people have existing antibodies against the Cas9 enzyme, which can lead to problems when attempting to use these enzymes as treatment methods (Yang et al., 2021).

4 Conclusion

CRISPR-Cas is a revolutionary gene editing system that has applications in several fields, including medicine, agriculture, and research. This system exceeds the abilities of existing gene editing technologies, such as ZFN, and TALENs, possessing considerably more benefits. CRISPR has a wide scope for treating a plethora of diseases, including cancer, and more specifically, bronchogenic carcinomas. It has several applications, including and not limited to the use of gene editing to create CRISPR screens, by identifying potential therapeutic targets such as oncogenes (EGFR, KRAS, and MET), and tumor suppressor genes. Additionally, CRISPR plays a crucial role in targeting lung cancer by inducing synthetic lethality in cryo-shocked tumor cells, as well as demonstrating the feasibility in T-cell modification. CRISPR is a groundbreaking tool that has the potential to be used to further improve and expand the existing treatment and therapeutic options in lung cancer. Although, there are several major benefits that can only be achieved by the highly efficient CRISPR-Cas system, there are key limitations that impact the overall efficacy of the tool that need to be taken into consideration, such as the off-target effects. Despite the fact that further research is crucial for the success of CRISPR, this discovery has transformed the fields of medicine and genetics. New discoveries are impending and CRISPR will only become more efficient, possessing the abilities to carry out previously

unthought of tasks in its therapeutic applications and beyond.

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