International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Unlocking the Potential of Dcas9: Epigenetic Targeting in Cancer Immunotherapy

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Abstract

Cancer's profound medical challenge underscores the urgency for innovative solutions. Contemporary immunotherapy has formidable challenges dealing with immune evasion, complex tumor microenvironments (TME), and checkpoint inhibitors. Improvements to immunotherapy have been made through genome editing for which the Cas9 system is utilized, but its reliance on DNA repair mechanisms coupled with elevated off-target effects presents limitations. One alternative approach to gene editing is the modulation of gene expression using dCas9 and associated effectors. An advantage is dCas9's non-cleaving nature which reduces the chance of off-target effects and avoids permanent changes to the genome, offering enhanced safety as a gene modulation tool. We reviewed ongoing studies and clinical trials on immunotherapy, gene expression, and dCas9 to address whether gene modulation via dCas9 could be a viable solution to improve gene therapy. In (one/a few) studies found that dCas9's adoption decreases off-target effects, heightening its appeal for improving immunotherapy compared to traditional gene editing with Cas9. Therefore, integrating dCas9 in suppressing key target genes, dcas9 with effector domains or epigenetic modifiers could prove as a pivotal strategy, enabling precise gene regulation and epigenetic modification. Specifically, transcriptional activators like VP64 and repressors like KRAB exhibit the potential to modulate target genes when used with dcas9. Acknowledging limitations, such as mutations affecting gene regulation, this study underscores the transformative potential of dCas9-mediated epigenetic regulation in reshaping the landscape of cancer treatment and calls for further investigation to unlock its full therapeutic potential.

Keywords: dcas9, Epigenetics, effector domains, immunotherapy, cancer

Introduction:

Cancer remains one of the leading challenges in modern medicine, impacting millions of lives worldwide and imposing significant health and socioeconomic burdens. The relentless growth and spread of abnormal cells, the hallmark of cancer, continues to confound researchers and clinicians alike. Despite remarkable strides in cancer research and treatment, the battle against this intricate disease is far from over. Cancer immunotherapy is a favorable approach for the treatment of various malignancies, this therapeutic strategy aims to stimulate or modulate immune responses, enabling the immune system to more effectively recognize and eliminate abnormal cells, pathogens, or dysfunctional components, ultimately contributing to improved health outcomes. While significant advancements have been made in the cancer immunotherapy field, limitations hinder its effectiveness. Checkpoint inhibitors, the suppressive tumor microenvironment influenced by immune-related genes, the reduced efficiency of CAR-T cell therapy due to altered gene expression [1], and errors in CRISPR due to the DNA repair mechanisms [2] are all

challenges associated with currently available immunotherapy. To address these challenges and enhance the effectiveness of cancer immunotherapy, innovative strategies are being explored, such as NK cell therapy [3], personalized vaccines [4], combination therapies [5], and the utilization of dCas9-mediated epigenetic regulation which this paper aims to highlight [6']

Checkpoint inhibitors have revolutionized cancer treatment by targeting immune checkpoints that maintain immune system balance [7]. These inhibitors, such as antibodies against programmed cell death protein 1 (PD-1) or its ligand PD-L1, unleash the immune system's ability to recognize and destroy tumor cells [8]. While checkpoint inhibitors have shown clinical efficacy in some cancer types like leukemia [9], they are limited by factors such as primary resistance, acquired resistance, and the lack of response in a substantial proportion of patients suffering from ovarian cancer [10], and lung cancer [11]. Thus, alternative strategies are needed to overcome these limitations and improve the overall effectiveness of checkpoint inhibitor therapy.

CAR-T cell therapy is another approach in cancer immunotherapy, particularly in the context of hematological malignancies. This therapy involves genetically modifying a patient's T cells to express chimeric antigen receptors (CARs) on their surface. These CARs are designed to recognize and bind to specific antigens expressed on tumor cells, leading to T cell activation, proliferation, and cytotoxicity against the tumor [12]. However, tumor cells frequently express immune checkpoint molecules such as PD-1, PD-L1, PD-L2, and CTLA4. These molecules form a natural regulatory mechanism that helps to prevent excessive immune responses and maintain immune tolerance. When immune checkpoint molecules on the tumor cells communicate with their corresponding receptors on the CAR-T cells (for example, PD-1 on CAR-T cells linking to PD-L1 on tumor cells), the immune response may be suppressed or restrained. This interaction effectively reduces the activity of CAR-T cells, allowing tumor cells to evade immune attack and potentially continue to grow. To address this problem, researchers have investigated combination therapies that involve using inhibitors (e.g., PD-1 or CTLA-4 inhibitors) to block these checkpoint molecules in addition to CAR-T cell therapy to improve the overall efficacy of CAR-T cell therapy in treating hematological malignancies and other cancers. [13]

In addition to the challenges posed by immune checkpoint molecules in CAR-T cell therapy, it's crucial to explore the impact of the tumor microenvironment (TME) on cancer immunotherapies. The TME consists of a complex network of cells, including tumor cells, immune cells, fibroblasts, and endothelial cells, as well as various signaling molecules and extracellular matrix components [14]. The TME can promote tumor growth, immune evasion, and resistance to therapy. Immune-related genes within the TME, such as the immune checkpoint proteins (e.g., PD-L1), cytokines (e.g., TGF-β), and chemokines (e.g., CXCL14), can exert suppressive effects on immune cells, impairing their anti-tumor functions [15]. TME, consisting of stromal cells, immune cells, and signaling molecules, plays a critical role in tumor progression and immune evasion. Altered expression of genes such as CXCL2, and IDO1 within the TME represses the activity of immune cells and promotes tumor growth and metastasis [16].

Epigenetic modifications play a crucial role in gene regulation and cellular identity, influencing gene expression patterns without altering the underlying DNA sequence [17]. These modifications involve chemical changes to DNA or histones, such as DNA methylation which silences genes by preventing transcription by the addition of methyl groups, and histone acetylation, which can impact the accessibility of genes to the transcriptional machinery [18]. Dysregulation of epigenetic marks and epigenetic genes is frequently observed in cancer, contributing to tumor initiation, progression, and immune evasion [19].

Gene therapy is a promising treatment for genetic disorders and diseases. A popular method is the use of Adeno-Associated Virus (AAV) vectors to deliver therapeutic genes into a patient's cells. However, AAV gene therapy has limitations due to its small packaging size, making it difficult to integrate efficiently into tumor cells [20]. Non-viral vectors, such as lipid-based and polymeric vectors, nanoparticles, and electroporation, are challenging to use in vivo due to their low gene transfection rate and cell-targeting capabilities [21]. Anti-tumor angiogenesis strategies involve down-regulating pro-angiogenic factors, increasing angiogenesis inhibitors' expression, and interfering with signaling pathways. While these techniques have shown improved outcomes for cancer patients in clinical trials, challenges persist due to imperfect gene transfer systems and the complexity of tumor angiogenesis. [22]

In recent years, dCas9, a modified version of the CRISPR-associated protein 9 (Cas9), has gained attention for its potential in epigenetic modulation [23]. Unlike the unaltered Cas9 protein that cleaves DNA, dCas9 has been altered to remove its cleaving domains, leaving it enzymatically inactive [24]. Despite this modification, it retains the ability to bind to specific DNA sequences via targeting with a short guide RNA. In addition, dcas9 can be fused with effector domains and epigenetic modifiers to precisely target genes or genomic regions of interest and modulate their expression, this study aims to explore the application of dCas9-based technologies in modulating gene expression to enhance the responsiveness of immunotherapies. By precisely manipulating the activity of genes or modifying epigenetic marks, I propose that we could improve tumor cell recognition by the immune system, enhance tumor antigen presentation, activate immune cells, and overcome immune evasion mechanisms employed by tumor cells. This research proposal outlines how dCas9 with effector domains can alter gene expression to enhance existing immunotherapy.

II. dCas9 Effectors: Tools for Epigenetic Modulation

A. Overview of dCas9 Structure and Function

dCas9 is a modified version of the CRISPR-associated protein 9 (Cas9). Unlike its counterpart, Cas9, which possesses DNA cleavage ability, dCas9'svcleaving domains RuvC and HNH are removed which removes cleaving activity [25]. Despite these modifications, dCas9 uses the traditional CRISPR-Cas9 system to recognize and cut specific DNA sequences at the target site via a guide RNA (gRNA) [26].

When a gRNA forms a complex with Cas9, it guides the Cas9 protein to the target DNA sequence through base-pairing interactions between gRNA and complementary DNA strands. Once the Cas9-gRNA complex binds to the target DNA sequence, Cas9 introduces a double-strand break (DSB) at that specific location. This process is an essential mechanism for modern genome editing and the introduction of specific changes in the DNA sequence [27]. However, most CRISPR-based editors capable of long-sequence knock-ins require single-strand nicks or DSBs, which can trigger the error-prone non-homologous end joining (NHEJ) pathways. This may result in variable efficiency and accuracy, leading to potential unwanted mutations in the genome [28].

To address this concern, scientists have developed dcas9 to modulate gene transcription along with decreasing off-target effects [29]. Instead of cleaving DNA, dCas9 retains its ability to bind to specific DNA sequences guided by a gRNA.

Upon reaching the target site, dCas9 does not induce DSBs but can modulate gene expression in a precise and controlled manner [30]. The gRNA guides dCas9 to the target site, and its fusion with effectors such as transcriptional activators, repressors, or epigenetic modifiers to the target genes allows the modulation

of gene expression. This opens up new possibilities for gene regulation and epigenetic modifications such as gene repression, gene activation, and epigenetic processes.

In short, dCas9 eliminates the risk of unintended mutations enhances the safety of gene regulation, and offers a new opportunity for epigenetic modulation. As a result, dCas9 has emerged as a viable option for targeted gene regulation and epigenetic modification with broad implications in various fields, including molecular biology, medicine, and biotechnology.

B. Fusion of dCas9 with effector domains or epigenetic modifiers

This approach holds the promise of finely tuning gene activity and epigenetic marks with precision, opening doors to potential therapeutic breakthroughs via modulation of gene expression. Drawing from a diverse pool of functional proteins, these effector domains equip dCas9 with specialized tools for targeted regulatory functions, shedding light on the complex mechanisms underlying gene expression and offering practical avenues for medical interventions. The fusion of dCas9 with effector domains or epigenetic modifiers is a key strategy in utilizing the CRISPR-Cas9 system for precise gene regulation and epigenetic modifications. Effector domains are functional regions derived from proteins that play specific regulatory roles in the cell. When fused with the dCas9 protein, these effector domains create complexes capable of precisely modulating gene expression or epigenetic marks.

Epigenetic modifiers are specialized proteins or enzymes that possess the ability to make subtle yet significant alterations to the marks attached to DNA or histone proteins. These marks, which don't change the actual sequence of DNA but influence how genes are activated or silenced, play a role in regulating gene expression. This process allows cells to respond and adapt to their environment without permanently altering their genetic code. Understanding the role of these modifiers in vivo has provided insights into how our genes are regulated and how their activity can be modulated [31].

Fusing transcriptional activator domains, like VP64, with dCas9 offers a potent means of augmenting gene expression. Transcriptional activator domains, such as VP64, can be fused to dCas9 to enhance gene expression. VP64, a well-known transcriptional activator domain, attracts the transcriptional machinery to the targeted gene's promoter region, leading to increased transcription and higher levels of the gene's product, such as mRNA or protein [32].

To assess the effectiveness of dCas9-VPR (VPR (Viral Promoter-Based Regulator is a complex that combines VP64, p65, and Rta transcriptional activators) in activating non-expressed or lowly-expressed genes, Fitzgerald et al. conducted an experiment involving the transfection of dCas9-VPR and guide RNAs (sgRNAs) targeting the promoter regions of Cnga1 or Opn1mw genes in cell lines lacking expression of one or the other. They found that a combination of three sgRNAs built on the effectiveness of gene activation observed in previous studies. [33]

Transcriptional repressor domains, like KRAB, can be fused with dCas9 protein to form a potent tool for repressing gene expression. dCas9-KRAB binds to DNA sequences using a guide RNA, similar to traditional Cas9 [34]. When it binds to the target gene's promoter, the KRAB domain attracts co-repressor proteins and chromatin-modifying enzymes, forming a repressive chromatin state that prevents transcription. The chromatin state involves modifications to histones, such as deacetylation and methylation [35], which compact the chromatin structure, making it less accessible to transcriptional machinery. This inhibits or blocks the assembly of RNA polymerase and other activator proteins, thus preventing transcription.

Utilizing effector domains provides a precise method for regulating specific genes, enabling both activation and repression based on the selected effector domain [36]. This sequence-specific targeting of regulatory proteins or complexes to genes of interest holds therapeutic potential.

Fusing dCas9 with effector domains or epigenetic modifiers precisely regulates genes and epigenetic marks. This creates a versatile toolkit for gene activation and repression with the potential for personalized therapies and shaping genetic research.

III. Modulating Gene Expression

A. Mechanisms of Modulation of Immune-Related Gene Expression

The advent of CRISPR-Cas9 technology has significantly advanced our ability to manipulate gene expression, making it a transformative tool in cancer research and therapy as it streamlined and expedited genetic research. Its user-friendly nature has empowered scientists across disciplines to unravel genetic mysteries, offering insights into disease mechanisms, developmental processes, and evolutionary history. The breakthroughs facilitated by CRISPR-Cas9 have not only accelerated our understanding of fundamental biological concepts but also fostered novel therapeutic avenues [37]. By combining dCas9 with effector domains, such as transcriptional repressors or activators, this strategy adeptly homes in on gene promoters, effectively enlisting the cellular transcriptional machinery and thereby amplifying gene transcription [38]. This targeted modulation of immune-related genes bears the potential to fortify the anti-tumor immune response, fostering the production of critical immune effectors, including cytokines, chemokines, and immune cell surface receptors.

This approach uses guide RNAs to direct dCas9 and effector domains to specific gene promoters, which activate or silence transcription of immune-related genes [39]. In 2014, Anthony K. et al. used TALE-TFs to enhance gene activation strategies in synthetic biology TALE-TFs consist of two distinct protein domains with specific functions: the repeat variable diresidue region, which binds to user-specified DNA sequences, and the VP64 effector domain, responsible for recruiting basal transcriptional machinery. They designed several TALE-TFs for genes linked to immunomodulation, inflammation, and cancer, including IL1RN, KLK3, CEACAM5, and ERBB2. Combinations of TALE-TFs resulted in idogistic effects on gene activation [40]. This method provides a means to investigate the role of specific genes and their epigenetic regulation in oncogenesis, offering valuable insights into the molecular mechanisms that drive cancer and potentially paving the way for targeted epigenetic therapies in the future.

In our exploration of gene control, the dCas9-KRAB complex plays a crucial role by focusing on gene promoters. Think of it as a molecular switch; the KRAB domain takes charge by blocking the assembly of transcriptional machinery. This strategic intervention holds promise for combatting the rampant overexpression of oncogenes—BCR-ABL, EGFR, KRAS (G12C), and HER2—commonly witnessed in tumor cells. It presents a focused approach to counteract these molecular drivers of cancer, curbing their influence on tumor advancement and progression. The dCas9-KRAB complex is directed to specific gene promoters, where the KRAB domain acts as a repressor [41]. This technique offers a focused means of inhibiting these oncogenes and curtailing their contribution to tumor development and progression.

This technique uses epigenetic control to suppress genes responsible for tumor growth and immune evasion. Multiplexed dcas9 effectors enable multiple combination strategies to achieve potent effects on tumor cells [42]. Targeting multiple genes or epigenetic marks implicated in tumorigenesis and immune regulation can reshape the tumor microenvironment and overcome common resistance mechanisms in cancer.

The CRISPR-Cas9 technology, specifically its dCas9 variant, can be used for cancer research and therapy. It can regulate gene expression and epigenetic marks, enhancing the immune response against tumors. By selectively turning genes on and off, this approach can reshape the tumor microenvironment and improve our understanding of cancer biology. This can lead to more effective personalized treatments.

B. Recruitment of Activators for Targeting Silenced Genes:

Transcriptional activator domains are functional regions derived from proteins that enhance gene expression by promoting the recruitment of RNA polymerase and transcriptional machinery to gene promoters. One such transcriptional activator domain is VP64, a well-known domain used in dCas9 effector systems. VP16 can also be fused with dCas9, but VP64 is preferred due to its stronger activation potential. Beerli et al. demonstrated this by engineering novel zinc finger proteins that bind specific DNA sequences and tested them for transcriptional activation using reporter constructs containing the erbB-2 gene's promoter regions. Both VP16 and VP64 fusion proteins led to successful gene activation, with VP64 resulting in a stronger stimulation. 4 fusion proteins led to significant 5-fold and 27-fold transcriptional stimulations, respectively. [43]

In the CRISPR-dCas9 system, the guide RNA (gRNA) directs dCas9 to specific genomic regions by binding to complementary DNA sequences adjacent to the target gene's promoter [44]. Once inside the cell, the dCas9-VP64 complex forms with the gRNA, precisely positioning it at the silenced gene's promoter. Here, the VP64 activator domain plays a pivotal role, recruiting the cellular transcriptional machinery, including RNA polymerase and co-activator proteins, to the promoter region [45]. This assembly enhances transcription initiation and elongation, resulting in increased mRNA transcript production.

Gene silencing represses gene expression, reducing mRNA and protein production. Silenced genes, often involved in cancer development, may be reactivated using transcriptional activators to restore normal cellular functions. Gene regulation research enhances our understanding of cancer biology and improves therapeutic approaches. Using transcriptional activators to modify genes and sensitize cancer cells to existing treatments is a suggestive avenue of investigation

Gene modulation has been successfully implemented in cancer treatment. Huisman et al. (2013) designed Artificial Transcription Factors (ATFs) to reactivate silenced genes in cervical cancer. One gene identified was C13ORF18, a potential tumor suppressor that was silenced due to hypermethylation. By using the potent transcriptional activation ability of the VP64 effector domain, the ATFs were able to specifically target the C13ORF18 gene promoter and reactivate gene expression. The ATF 1ab-VP64 showed efficacy in rejuvenating C13ORF18 in methylated HeLa cells [46].

Recently, Morita et al. (2020) conducted a study to improve gene expression in the body. They combined dCas9 with a transcription activation domain to create dCas9-VP64, identifying VP64 as the most effective partner to work with TET1 in the dCas9-SunTag setup. They used a single-guide RNA (sgRNA) to coordinate the interaction between VP64 and TET1 and successfully activated target genes. VP64's synergistic effect was observed in eight out of ten examined genes [47].

Continuing our exploration, it becomes evident that several other genes exhibit potential as targets for the transcriptional activator VP64. Compromised expression of these genes can contribute to cancer development. Targeting these genes can improve cancer therapy by restoring cellular functions and making cancer cells more sensitive to treatments that are already established.

MGMT (O6-Methylguanine-DNA Methyltransferase) [48]

CHFR (Checkpoint with Forkhead-Associated and Ring Finger Domains) [49]
FANCF (Fanconi Anemia Complementation Group F) [50]
BRCA1 (Breast Cancer Susceptibility Gene 1) [51]
MLH1 (MutL Homolog 1) [52]
GSTP1 (Glutathione S-Transferase Pi 1) [53]
TGM2 (Transglutaminase 2) [54]
To summarize, CRISPR-dCas9 provides high specificity in targeting silenced genes via precise complementarity between gRNA and target DNA. It enables temporal and spatial control over gene activation and has potential therapeutic applications. Targeting silenced genes with transcriptional activators can slow down tumor growth, induce cancer cell death, and enhance the body's ability to combat infections or autoimmune disorders.

C. Utilization of repressors to silence genes

Gene silencing is a crucial process in regulating gene expression, and repressor domains play a pivotal role in inhibiting gene expression by preventing or blocking the assembly of the transcriptional machinery at gene promoters. The CRISPR-dCas9 system can harness the power of repositories, such as Krüppel-associated box (KRAB), to achieve targeted gene silencing [55].

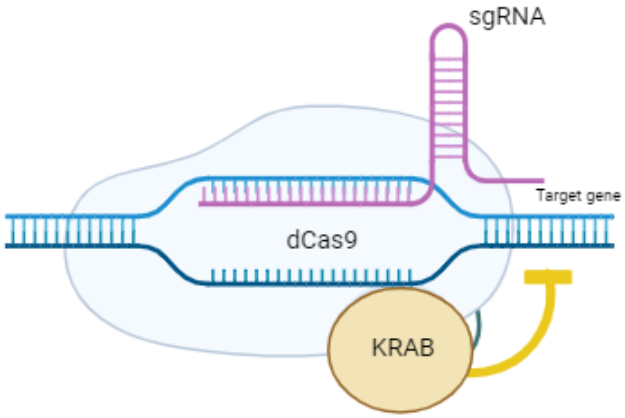

Figure 1: The following figure illustrates the use of dcas9 for target gene suppression by using the effector domain KRAB

The mechanism of action of the dCas9-KRAB system involves guiding the dCas9 effector to the target gene using specific guide RNA (gRNA). Once bound to the target gene's promoter region, the KRAB repressor domain interacts with co-repressor proteins and histone-modifying enzymes, leading to the formation of heterochromatin. Heterochromatin is a tightly packed chromatin structure that renders the gene's promoter region inaccessible to the transcriptional machinery, effectively silencing the gene.
Importantly, the epigenetic modifications facilitated by the dCas9-KRAB complex, including histone deacetylation and histone methylation, lead to structural changes in the chromatin, creating an inhibitory environment that obstructs gene transcription. This epigenetic silencing ensures that the gene remains dormant and prevents its expression.
Furthermore, a study, by Beerli et al. (1998) generated zinc finger proteins that bind to previously untargeted DNA sequences using predefined domains, resulting in gene-specific polydactyl proteins

capable of repressing gene expression. The KRAB domain, in particular, was found to be the most effective transcriptional repressor compared to ERD or SID, and it can inhibit both TATA-dependent and TATA-independent transcriptional initiation of the promoter. This study highlights the versatility of engineered transcription factors in achieving graded levels of gene repression. [56]

The potential of the dCas9-KRAB system in gene silencing is evidenced by its ability to target various disease-associated genes, such as oncogenes like BCR-ABL, EGFR, KRAS(G12C), HER2[57], epigenetic genes such as EZH2, ARID1A, IDH1[58], immune-related genes like PD-1, PD-L1, PD-L2, CTLA4[59], as well as other genes like MUC1[60], and FANCF[61] are potential targets. Selectively guiding dCas9-KRAB to the promoter regions of these genes, can effectively downregulate their expression and potentially alleviate disease phenotypes.

In 2023, a study by Roy Choudhury et al. demonstrated a new approach to fight cancer by inhibiting the S100B gene, known to drive the development of melanoma cells. The researchers used a combination of dCas9-KRAB fusion and S100B-specific sgRNAs to target the regulatory regions of the gene in murine B16 melanoma cells. This resulted in a significant reduction in S100B mRNA levels, leading to reduced cell viability and increased susceptibility to cisplatin. The study highlights the potential of this approach for targeted gene suppression in melanoma cells. [62].

Casting our gaze toward therapeutic prospects, the utilization of dCas9-KRAB for gene silencing presents significant potential in therapeutic applications. One of the key advantages of this approach is its potential to target and silence genes that are overexpressed in various diseases, including certain types of cancer. Guiding dCas9-KRAB to the promoter regions of oncogenes or disease-associated genes can effectively downregulate their expression, which may slow down tumor growth or induce cancer cell death. This strategy opens up novel avenues for cancer therapy and may hold the potential for treating other genetic disorders caused by gene overexpression.

IV. Enhancing Responsiveness to Immunotherapies

A. Importance of Modulating Immune-related Genes and Epigenetic Marks in Tumor Cells

In the evolving field of cancer immunotherapy, significant strides have been made thanks to the use of the body's immune system to fight cancer. However, there are complex challenges that need to be addressed to continue developing effective treatments. One of the main issues is the interactions among immune-related genes, which present barriers to checkpoint inhibitors. Moreover, the genetic dynamics of the immune system play a crucial role in the tumor microenvironment, and even slight imbalances can reduce the effectiveness of treatments. CAR-T cell therapy is another approach that relies on precise gene control, and any imbalances can significantly impact its ability to combat cancer cells. This section aims to shed light on these challenges, explaining their mechanisms and consequences. Although these challenges pose barriers, they also inspire researchers and clinicians to decipher their intricacies, paving the way for personalized and potent cancer immunotherapies.

The tumor microenvironment (TME) presents various challenges for immune evasion and immune-related genes by promoting tumor growth, invasion, and immune evasion [63] affecting the makeup and behavior of immune cells within the TME [64]. Epigenetic regulation using dCas9 can create a more favorable immunosuppressive microenvironment, which promotes anti-tumor immune responses. Epigenetic modifications can also modulate metabolic pathways in tumor cells, which can influence the anti-tumor immune response

Immune evasion is a persistent challenge that is mainly driven by the use of cell surface markers. The expression of these markers on cancer cells plays a critical role in their ability to evade the immune system. One such marker is the Ig receptor, PD-L1 and PD-L2, which interact with PD-1. When PD-L1 on the cancer cell surface binds to PD-1 on T cells, it sends a signal to the T cells to become "exhausted" or "dysfunctional." This exhaustion results in decreased T-cell activity, leading to the suppression of the immune response against the cancer cells. PD-L1 is present in both hematopoietic and non-hematopoietic cells, and its expression is controlled by interferon-gamma (IFN-γ) and cytokines, including IL-7 and IL-15[65]. The PD-1/PD-L1 pathway hinders T cell activation, proliferation, and cytotoxicity in the tumor microenvironment (TME). The level of PD-L1 expression is closely associated with the effectiveness of anti-PD-1/PD-L1 therapy in specific cancers like non-small cell lung cancer, melanoma, and others [66]. PD-L2, found on the surface of DCs, macrophages, mast cells, and some B cell groups, is highly expressed in the TIME of renal cell carcinoma (RCC) and lung squamous cell carcinoma (LUSC). The concurrent presence of PD-L1 and PD-L2 in tumor cells can substantially hinder the anti-tumor immune response [67]. These insights emphasize the vital need to overcome this challenge. Understanding the complexities of immune checkpoint regulation is crucial to fully harness the potential of cancer immunotherapy.

Gene	Function	Expression
IFN-γ [68]	promote macrophage activation, mediate antiviral and antibacterial immunity, orchestrate activation of the innate immune system, coordinate lymphocyte–endothelium interaction, regulate Th1/Th2 balance, control cellular proliferation and apoptosis, and increase expression of cancer-related genes	Overexpressed
IL-7[69]	Promotes cytokine secretion, activates antitumor activity	Under expressed
IL-15 [70]	Enhances immune response, toxicity, NK cell development	Under expressed
IL-2 [71]	Lymphocyte regulation, increases toxicity, sustains T cell response	Under expressed
IL21 [72]	Regulates T cells,	Under expressed
PD-L2, [73]	T Cell exhaustion,	Overexpressed

Gene	Function	Expression
PD-L1 [74]	Inhibition of T Cell proliferation, immune evasion	Overexpressed
TGF-β [75]	Immune evasion	Overexpressed
IDO1 [76]	Immune evasion	Overexpressed
CXCL14 [77]	Immunosuppressant	Overexpressed
HLA [78]	Immune surveillance	Under expressed
EZH2 [79]	Tumor progression	Overexpressed
FANCF [80]	DNA damage repair	Under expressed
MGMT [81]	DNA damage repair	Under expressed

Table 1 =Table 1 provides a comprehensive depiction of genes, including their associated functions, and expression status in cancer patients compared to a control or reference condition. Gene expression levels are categorized as either "Overexpressed" or "Under-expressed," shedding light on the role of these genes in the context of cancer.

The intricate regulation of immune-related genes and epigenetic marks is critical in modulating the tumor microenvironment and influencing the immune response to cancer. Dysregulation of these genes and modifications can lead to tumor cells evading detection and destruction by the immune system through immune evasion mechanisms. Therefore, there is a growing interest in targeting and modulating immune-related genes and epigenetic marks in tumor cells as potential strategies to enhance the effectiveness of cancer immunotherapy.

Effective cancer immunotherapy focuses not only on eliminating existing tumors but also on establishing immune memory to prevent tumor recurrence. Modulating immune-related genes involved in memory T cell formation and maintenance can significantly contribute to long-term immune surveillance and improved patient outcomes.

In conclusion, this exploration has highlighted the challenges of immune evasion in cancer immunotherapy. Immune checkpoint molecules impede T cell function, but dCas9-based interventions offer promise. By upregulating genes and inhibiting immunosuppressive factors, dCas9 can counteract tumor-induced immune suppression, foster anti-tumor immune responses, and amplify cancer immunotherapy's effectiveness. These interventions hold great potential for reshaping cancer treatment and elevating patient outcomes

B. Potential Impact on Tumor Cell Recognition by the Immune System:

Tumor antigen presentation stands as a key player in this genetic interaction, guiding a sequence of events that bridges the gap between tumor cells and the immune system [82]. Within tumor cells, proteasomes break down intracellular proteins into smaller peptide fragments, encompassing both normal cellular proteins and distinct abnormal or mutated proteins unique to cancer cells. These peptide fragments, known as antigens, intricately bind to specialized molecules called Major Histocompatibility Complex (MHC)

proteins, wherein MHC class I molecules exhibit antigens from the cell's cytoplasm, and MHC class II molecules present antigens from endosomes or lysosomes. Subsequently, the MHC-antigen complex journeys to the cell surface for display. Vigilant immune cells, particularly T cells, continually survey the cellular landscape, engaging with the presented antigens on tumor cells. T cells are equipped with receptors known as T cell receptors (TCRs), designed to recognize specific antigens showcased by MHC molecules. The congruence of TCRs with presented antigens prompts an immune response, propelling the activation of T cells. Transformed into effector cells, these activated T cells acquire the ability to target and eliminate tumor cells displaying the antigen of interest, thus fostering a critical immune response that holds the key to cancer control and eradication.

Tumor cells frequently downregulate major histocompatibility complex class I (MHC-I) molecules, leading to reduced recognition by cytotoxic T cells [83]. With precise targeting and upregulation of MHC-I-related genes, dCas9-based strategies hold the capability to amplify tumor cell visibility to immune cells, triggering a stronger immune response against cancer cells.

Through dCas9-based approaches, MHC class I-related genes, such as HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, and HLA-G, can be targeted to increase their expression [84, 85]. Upregulating MHC class I molecules enhances tumor cell visibility to immune cells, triggering a stronger immune response against cancer cells. Notably, HLA-F is often downregulated in cancer, providing an avenue for targeted modulation.

Immune Cell Activation

Epigenetic regulation using dCas9-based technologies can activate immune response-related genes, such as cytokines, chemokines, and costimulatory molecules by promoting the expression of these genes, the tumor microenvironment becomes more favorable for immune cell activation and infiltration.

For instance, cytokines like IL-2, IL-7, IL-15, and IL-21 play crucial roles in the expansion of NK cells and T lymphocytes [86]. However, in cancer, these cytokines are often under-expressed, hampering the anti-tumor immune response. Developing drugs based on cytokines requires careful fine-tuning of their pharmacological properties using biotechnological strategies [87]. By using dCas9 to modify the expression of cytokines, cytokine-based cancer immunotherapy can be fine-tuned, ensuring improved pharmacological properties and reduced toxicities that are caused by traditional methods by administering high doses of interleukins[88].

Chemokines, another group of immune response molecules, direct the migration and positioning of immune cells throughout the body. However, certain chemokines like CXCL14 are silenced in cancer due to promoter region methylation [89]. Dcas9, along with transcriptional activators can demethylase CXCL14, potentially restoring its expression and enhancing immune cell migration to cancer sites.

As we delve deeper into the subject of immune evasion, we encounter the complex tactics that tumors employ. Immune evasion factors such as the enzyme IDO, the cytokine TGF-β, and immune checkpoint molecules PD-1 and PD-L1 stand as formidable obstacles to successful immunotherapy. Nonetheless, dCas9's targeted epigenetic modifications offer a practical avenue to counter these evasion mechanisms. By directly tackling these challenges, dCas9-based interventions hold the potential to considerably boost immune recognition and activation, ultimately paving the way for more effective cancer treatments and improved patient outcomes.

IDO (Indoleamine 2, 3-Dioxygenase):
IDO is an enzyme expressed by tumor cells and immune cells within the tumor microenvironment. It plays a crucial role in immune evasion by degrading the essential amino acid tryptophan [90]. Depletion of tryptophan inhibits T cell activation and promotes the generation of immunosuppressive Tregs, contributing to immune tolerance and tumor growth. High expression of IDO has been observed in various tumors, including melanoma [91], colon cancer [92], brain tumors [93], and ovarian cancer [94], therefore is a target for epigenetic silencing. The intricate interactions of IDO with immune checkpoints such as CTLA-4 and PD-1 are not yet fully elucidated, but they present potential opportunities for targeting multiple immune evasion pathways simultaneously.
While the application of dCas9 in modulating IDO expression is yet to be fully realized, prospective routes for investigation are emerging. Epigenetic regulation harnessed through transcriptional repressors like KRABs offers a plausible approach to manipulate IDO expression and impact the intricate immune evasion mechanisms governed by this enzyme. As the scientific journey unfolds, the potential of dCas9 to unravel the complexities of IDO's role in immune evasion underscores the transformative potential of genetic intervention in advancing cancer immunotherapy.

TGF-β (Transforming Growth Factor-Beta):
TGF-β is a cytokine that plays a critical role in immune suppression. Within the tumor microenvironment, TGF-β inhibits the function of immune cells, including T cells and natural killer (NK) cells, thereby reducing their ability to recognize and attack cancer cells. Additionally, TGF-β promotes the differentiation of regulatory T cells (Tregs), which further dampens the immune response [95]. Recent research by Rayana et al. in 2021, has explored the use of CRISPR interference, specifically employing the KRAB-dCAS9 (Krüppel-associated box domain fused to deactivated Cas9), to suppress TGF-β expression in the trabecular meshwork of the eye, associated with primary open-angle glaucoma and elevated intraocular pressure. This study demonstrated that the CRISPR interference system with KRAB-dCAS9 effectively inhibited TGF-β2 expression, suggesting the potential of this approach for treating TGF-β2-induced ocular hypertension associated with glaucoma. This highlights the potential of dCas9-mediated epigenetic regulation to target TGF-β and counteract its immunosuppressive effects in cancer [96].
In conclusion, overcoming immune evasion mechanisms through dCas9-mediated epigenetic regulation holds tremendous promise for enhancing the effectiveness of cancer immunotherapy. Targeting immunosuppressive factors like IDO and TGF-β has the potential to pave the way for novel therapeutic strategies that improve immune recognition and activation, leading to better outcomes for cancer patients.

Immune Checkpoint Modulation:
Immune checkpoint molecules, such as PD-1 and PD-L1, are frequently overexpressed in tumor cells, contributing to immune evasion and inhibiting immune responses [97]. dCas9-mediated epigenetic regulation presents a promising avenue for targeting these checkpoints, promoting their downregulation, and potentially enhancing the effectiveness of checkpoint inhibitor therapies.
Checkpoint inhibitors, like antibodies against programmed cell death protein 1 (PD-1) or its ligand PD-L1, play a critical role in unleashing the immune system's ability to recognize and destroy tumor cells. These inhibitors block the interaction between immune checkpoint molecules, preventing the suppression of immune responses and facilitating the immune system's recognition and attack of cancer cells in

combination with CAR-T cell therapy, checkpoint inhibitors have shown promise in enhancing efficacy while minimizing systemic toxicities [98]. Additionally, engineering CAR-T cells to secrete immune checkpoint inhibitors or counteract immunosuppressive factors in the tumor microenvironment, such as prostaglandin E2 (PGE2) and reactive oxygen species (ROS), as well as inhibiting cytokines like TGF-β and IL-6, has improved tumor infiltration and overcome immunosuppression [99].

Preclinical studies have provided evidence of the potential of dCas9-mediated gene regulation in improving the efficacy of checkpoint inhibitors. Through dCas9-SunTag-sgARRAY-mediated labeling, endogenous KAT8 and IRF1 condensation at the PD-L1 promoter was observed, providing insight into dCas9's direct involvement in orchestrating PD-L1 expression through biomolecular condensates, offering potential avenues for precise immunotherapy strategies. [100,101].

In summary, dCas9-based techniques hold significant potential for advancing cancer immunotherapy. By improving immune cell recognition, tackling immune evasion mechanisms, and fine-tuning immune checkpoints, dCas9 offers a practical pathway to enhancing cancer treatment strategies. This technology brings us closer to more effective approaches for combating cancer and improving patient results.

V. Mutations and Limitations

Advancements in cancer immunotherapy have led to the emergence of dCas9-based gene regulation as a favorable approach for precise epigenetic editing and overcoming immune evasion mechanisms. However, like any new technology, there are challenges to overcome. One of the major factors that can affect the success of dCas9-mediated therapies is genetic mutations in target genes. These mutations can significantly impact the effectiveness of dCas9-based treatments, and thus require careful consideration and innovative solutions [102]. This section aims to explore how genetic mutations affect cancer immunotherapy, with a focus on dCas9-mediated gene regulation. By doing so, we hope to shed light on the way forward towards more personalized and effective cancer treatments.

1. Impact of Genetic Mutations:

Genetic mutations play a pivotal role in cancer development and progression, and they can significantly impact the effectiveness of dCas9-mediated gene regulation in cancer immunotherapy. Understanding the influence of mutations on dCas9-based therapies is essential to harness their full potential for precise epigenetic editing and overcoming immune evasion mechanisms.

Genetic mutations in target genes present significant challenges to dCas9-mediated gene regulation. Mutations in gene promoter regions or enhancer elements can alter the binding of dCas9 and its effectors, reducing their ability to target and modify specific sites [103]. Cancer cells commonly accumulate genetic mutations and chromosomal abnormalities, including translocations, deletions, inversions, and gene amplifications [104]. These chromosomal changes complicate cancer genomes, affecting how genes are organized and controlled. When using dCas9 for gene regulation, the presence of these chromosomal abnormalities in cancer cells adds complexity. The altered chromatin structure due to these abnormalities can make it harder for dCas9 to access target sites, potentially affecting its regulatory effects. Consequently, the interplay of mutations, chromosomal abnormalities, and their impact on dCas9's function significantly influences its effectiveness in precise gene regulation. Additionally, specific mutations might even lead to the loss of dCas9 binding sites, limiting the feasibility of accurate epigenetic editing.

For instance, in cancer, mutations in critical genes like TP53, PTEN, ERCC5, and IDH1 are highly enriched [105]. BRCA1, which is essential for DNA damage repair through homologous recombination, can undergo mutations that impair this repair mechanism, leading to genomic instability and increased cancer risk, especially in ovarian and breast cancer [106]. Similarly, TP53, known as the "guardian of the genome," and involved in DNA damage repair is frequently mutated in various cancers, disrupting the cell cycle regulation, DNA repair, and apoptosis [107,108].

Moreover, mutations in cancer cells can also affect the efficacy of checkpoint inhibitor therapies, which rely on blocking immune checkpoint molecules like PD-1 and PD-L1. Frequent TP53 somatic mutations can disrupt the DNA damage response and apoptosis pathways active in many early-stage cancers [109].

In the complex interplay of genetic mutations and dCas9-mediated gene regulation, a deeper understanding is essential to fully realize the potential of cancer immunotherapy. Researchers and clinicians seek innovative solutions to counteract mutation-driven challenges, demonstrating our commitment to conquering cancer complexities for improved, personalized treatments.

2. Off-Target Effects:

In addition to the challenges posed by mutations, dCas9-based therapies may encounter off-target effects. Despite efforts to improve specificity, dCas9 and its effectors may unintentionally bind to off-target genomic regions, leading to unintended gene regulation [110]. Minimizing off-target effects is critical to ensure the safety and precision of dCas9-mediated epigenetic regulation.

3. Epigenetic mechanisms

Epigenetic changes play a significant role in cancer development and progression [111]. These changes are DNA methylation, histone modifications, chromatin remodeling, and non-coding RNAs, which can lead to abnormal gene expression patterns [112,113]. While dCas9-based approaches hold promise for modulating gene expression, overcoming the challenges posed by existing epigenetic mechanisms is essential to effectively harness their potential.

The application of dCas9-based techniques encounters several challenges when dealing with the intricate landscape of epigenetic changes in cancer. Firstly, the precision of dCas9 targeting specific gene promoters, pivotal for effective modulation of gene expression, is hindered by the altered epigenetic environment in cancer [114]. This variation affects the accessibility of these promoters, impeding dCas9's ability to bind and exert its intended effects. Additionally, achieving the appropriate dosage of dCas9-based regulators is critical; excessive activation or repression can disrupt the delicate gene expression balance, potentially leading to unintended consequences. The heterogeneity of epigenetic profiles within cancer cells further complicates therapy implementation [115]; effectively targeting all cells with dCas9-based interventions becomes challenging when epigenetic states vary significantly. Additionally, cancer cells often develop resistance mechanisms against dCas9 interventions, potentially reinstating epigenetic marks or activating compensatory pathways to counter the treatment [116]. The multifactorial nature of epigenetic changes further amplifies the challenge; the intricate interplay of diverse regulatory layers makes targeting a single aspect insufficient to fully address the overall epigenetic dysregulation in cancer.

To address the challenges posed by existing epigenetic mechanisms in dCas9-based therapies for cancer, several strategies show promise. Enhanced targeting methods, like modified gRNAs or epigenetic reader domains, could guide dCas9 more effectively to sites with altered epigenetic marks, improving its binding and impact [117]. Additionally, tailoring dCas9-based treatments to individual patients based on their

unique epigenetic profiles could maximize therapy efficacy. Lastly, optimizing delivery methods would ensure efficient dCas9 delivery to target cells, enhancing its therapeutic potential [118].

VI. Conclusion

The research paper discusses the groundbreaking potential of dCas9-based technologies as a precise and safer alternative to traditional CRISPR-Cas9 systems. This technology unlocks the unique power of epigenetic modulation to enhance cancer immunotherapy. By ingeniously removing the cleaving domain of Cas9, dCas9 offers a method for targeted gene regulation without altering the DNA sequence itself, providing unprecedented control over gene expression. Throughout the study, the paper highlights the remarkable capabilities of dCas9-mediated gene regulation, particularly in bolstering the expression of immune-related genes in tumor cells. The fusion of dCas9 with transcriptional activator domains, such as VP64 and VP16, enables researchers to precisely target gene promoters and amplify gene expression, thus fortifying the anti-tumor immune response. The paper also highlights the power of repressor domains, such as KRAB, in gene silencing, enabling the selective inhibition of certain genes implicated in cancer and other diseases. The impact of dCas9-mediated gene regulation on tumor antigen presentation and immune cell activation within the tumor microenvironment is significant. By modulating the expression of immune-related genes and epigenetic marks, dCas9 enhances MHC molecule expression and optimizes the presentation of tumor antigens. This innovative approach empowers immune cells, particularly T cells, with heightened efficacy in recognizing and targeting cancer cells, unshackling the body's innate defense mechanisms against cancer. Furthermore, the paper underlines the potential of dCas9-mediated gene regulation in disrupting cancer's evasion tactics. Immune evasion mechanisms employed by cancer cells are disrupted as dCas9 inhibits immunosuppressive factors and downregulates immune checkpoint molecules such as PD-1 and PD-L1. The strategic combination of dCas9-based therapies with checkpoint inhibitors results in a synergistic effect, fostering a robust and enduring anti-tumor immune response. The paper also acknowledges the challenges posed by genetic mutations in target genes that could hinder dCas9's efficacy. Understanding the influence of these mutations is essential to optimize the precision and effectiveness of dCas9-based therapies in diverse cancer types and patient populations. In conclusion, to harness the full capabilities of dCas9-mediated gene regulation, limitations must be addressed. Minimizing off-target effects and devising efficient delivery methods for tumor cells are paramount to ensure safety and precision. Furthermore, taking epigenetic mechanisms into account is critical for sustained treatment efficacy and success.

Acknowledgments:

I would like to express my heartfelt gratitude to the following individuals and institutions who have played an integral role in the success of this research project:

Lumiere Education: am sincerely thankful to Lumiere Education for generously providing me with a full scholarship to their individual summer research program. This opportunity not only supported my academic journey but also allowed me to delve into this research project.

Segovia Garcia: I am indebted to my personal mentor, Segovia Garcia, who provided unwavering guidance and support throughout this project. Their expertise and mentorship were invaluable in shaping the trajectory of my research.

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