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A Comparative Analysis of CRISPR Screening Technologies

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Abstract

This paper offers a general review and comparative analysis of various types of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technologies. It evaluates the strengths and weaknesses of these technologies to identify the optimal approach for conducting genetic screens. Through an extensive literature review, this paper examines CRISPR nuclease, CRISPR activation (CRISPRa), and CRISPR interference (CRISPRi) screens. This study concludes that CRISPRa and CRISPRi are more advantageous due to their use of deactivated Cas9 proteins that only over-express or deactivate genes rather than irreversibly breaking genes like CRISPRn. Notably, CRISPRa is unique in its ability to over-express genes, while the other two technologies deactivate genes. Future studies may focus on inducing multiple mutations simultaneously—both gain-of-function and gene knockout—to carry out a more complete screen that can test the combinatorial effect of genes. Likewise, targeting both exons and introns can offer a more thorough understanding of a specific phenotype.

Keywords

CRISPR, CRISPR-Cas9, Genome Engineering, sgRNA, Genetic Screen

1. Introduction

Genetic screens are widely used methods to identify genetic functions and analyze biological pathways. In essence, these screens induce a mutation and subsequently link it with a phenotype of interest. Typically, researchers induce a range of mutations on different loci of DNAs into cells. Following this, they observe the consequent phenotypic changes from these mutations. By honing in on a specific resultant phenotype, researchers can determine the corresponding DNA mutation responsible for it. To affirm these discoveries, known mutations will be

inserted to confirm their phenotypical effects. Genetic screens have notably influenced modern science since they not only reveal genetic functions but also advance disease treatments. By pinpointing mutations leading to a certain disease, genetic screens aid in locating the disease's genetic cause and identifying potential therapeutic targets. Moreover, these genetic screens can address challenges like drug resistance, underscoring their value in healthcare by assessing drug efficacy.

While several methods exist for genetic screens, such as RNA interference (RNAi), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based technologies have taken precedence, particularly owing to their precision in targeting genes.

RNAi is a commonly used method to conduct gene knockdown by using a siRNA complementary to the gene of interest. The siRNA then binds with the RNA induced silencing complex, causing the degradation of the targeted mRNA and thus blocking the protein-making process. As a more recently discovered technology, CRISPR's origins trace back to the immune system of bacteria. Researchers discerned short-term repetitive DNA sequences in bacterial genomes, and they identified the interspersed sequences in between the repeats as the sequences originated from bacterial phages. These sequences are termed CRISPR sequences. Bacteria utilize these CRISPR sequences to recognize and break bacterial phages when they are infected. Scientists thus can appropriate the system to alter the expression of target genes, which is useful for genetic screens. Adapting this system for human applications, researchers take the CRISPR sequences from bacteria to produce the Cas9 protein, which cleaves DNA to induce mutations, thus altering gene expression. These two common methods show their strengths and weaknesses. The major drawbacks of RNAi are its inability to target non-coding genes and its substantial off-target effects [1]. Boettcher and Mcmanus' review of different gene knockdown tools evaluates the accuracy and suited application for each tool. The RNAi system naturally occurs in the cytoplasms, so the non-coding genes in the nucleus cannot be efficiently targeted. Moreover, the siRNA can knock down non-targeted genes when the genes have only a limited sequence complementary to it, causing a severe off-target effect that alters the phenotype. On the other hand, CRISPR's off-target effect is not likely to cause an undesired phenotype because the dCas9 which is used to cut the gene would be limited to the targeted area. This paper will focus on CRISPR, which has great potential due to its wide targeting range and target specificity. Over time, numerous forms of the CRISPR system have been developed, including CRISPR nuclease (CRISPRn), CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi), each presenting its own set of advantages and shortcomings.

While a plethora of studies have employed a single CRISPR technology for specific biological pathways or genome-size screens, holistic investigations remain scant. This paper assesses diversified cases and technologies, aiming to guide

the optimal screening approach for various medical challenges. Generally speaking, CRISPRi and CRISPRa demonstrate greater versatility compared to CRISPR nuclease, with CRISPRa exclusively enabling gain-of-function of genes.

2. Types of CRISPR

2.1. CRISPR Nuclease

CRISPR nuclease represents the traditional approach in CRISPR-based gene editing, in which researchers employ a Cas9 protein complex to cut the gene and thereby induce the loss-of-function of a gene. In this system, the complex executing gene knockout comprises single guide RNA (sgRNA) and the Cas9 protein. The Cas9 protein functions as an RNA-directed endonuclease that requires an RNA guide to locate the target DNA sequence so that it can accurately snip the DNA. Thus, a specifically designed sgRNA is employed, consisting of a constant region that binds to the Cas9 protein and a programmable region complementary to the target DNA sequence in each case (Cui *et al.*, 2018) [2]. The study conducted by Cui *et al.* offered a review of sgRNA design tools that explained the process of targeting and cleavage. To initiate the process, researchers introduce both the sgRNA sequence and the sequence coding for the Cas9 protein into the target cell, where they are transcribed and translated into an enzyme complex. This complex subsequently binds to the target DNA sequence and creates a double-strand break. Cellular machinery then commences either homology-directed repair (HDR) or non-homologous end joining (NHEJ). In the case of HDR, a DNA template can be introduced to fill the broken gaps. However, when trying to knock out a gene, these DNA templates are unable to be introduced into the cell. In the absence of an introduced DNA template, the cell performs NHEJ, culminating in loss-of-function of a gene (Jiang & Doudna, 2017) [3]. The ability to selectively knock out a wide range of genes allows researchers to determine which genes are involved in the expression of an interested phenotype.

2.1.1. CRISPRn Screen for Genes Associated with Lung Metastasis

One illuminating study employed a genome-wide CRISPR-Cas9 gene knockout strategy to identify genes implicated in primary tumor growth and subsequent metastasis (Chen *et al.*, 2015) [4]. Earlier studies had failed to delineate the pathway connecting primary tumor size with metastasis. In this regard, the CRISPR technology permitted precise gene knockouts, superseding previous methodologies that only allowed random insertions of mutation.

In this study, a library of 67,405 sgRNAs was used to induce mutations in a non-metastatic cell line of mice. Following weeks of vitro culture, lung metastasis was observed in 89% of the sgRNA-transduced mice, compared to 0% in the non-transduced control group. To investigate the representation of specific genes in cellular proliferations, researchers measured the depletion level of sgRNA. Within the late-stage primary tumors, only less than 4% of the sgRNAs from the

primary library were detected; this number further reduced to just 0.4% of in the metastases. The difference in sgRNA library dynamics in different stages of cancer indicated distinct genes critical to each stage of cancer development. Among the cells induced with sgRNAs, some shows the phenotype of becoming late stage tumors, so researchers trace the RNA sequence of these late stage tumor cells to pinpoint the specific sgRNAs induced into these cells. The most enriched sgRNAs are most related to the phenotype. The genes targted by the most enriched sgRNAs are thus the disease-causing genes. By this way, researchers identified 24 genes targeted by more than two of these enriched sgRNAs. The validity of these findings was corroborated by patient data, which showed mutations in these 24 genes in numerous cancer cases.

Among these 24 candidates were known tumor repressors such as Pten and NF2. For instance, NF2 encodes to produce the protein Merlin, which controls the pathway associated with cell growth and division. Therefore, the expression of NF2 inhibits uncontrolled cell proliferation. Interestingly, the study suggested that the NF2 may play a more active role in promoting metastasis rather than in primary-stage tumors. To validate these findings, researchers transduced the sgRNAs targeting these candidates into mice. The in vivo experiments demonstrated that the loss of functions in these genes indeed facilitated metastasis. This study therefore identifies genes that promote tumor growth and metastases, which is rarely studied as most studies focus on genes that cause primary-stage tumors, so the results of this study have the potential to aid the inhibition of tumor growth and late-stage tumors.

2.1.2. CRISPRn Screen for PLX Resistance

Vemurafenib (PLX), a BRAF protein kinase inhibitor, is commonly used in melanoma treatments to inhibit the growth of mutated cells. However, acquired resistance to PLX often poses a clinical challenge. To address this issue, researchers conducted a genome-scale screen to identify genes that could confer PLX resistance such as NF1 and NF2 (Shalem *et al.*, 2014) [5].

Targeting 18,080 genes found in human exons, researchers designed 3 to 4 sgRNAs for each gene, thereby assembling a comprehensive sgRNA library. Utilizing a Lentiviral vector, researchers transduced the Cas9 complex into the cells to eliminate the need to generate a cell line specifically for Cas9 complex expression. 14 days post-transduction, the composition of the remaining sgRNA content in the sample had diverged significantly from the original library. Researchers subsequently identified the enrichment of several sgRNAs, revealing that their knockout was a causation of PLX resistance. Previously known genes related to PLX resistance—NF1 and MED12—were identified, thereby validating the accuracy of this screen, along with previously unknown genes including NF2 and CUL3.

2.2. CRISPR Activation

CRISPR activation, or CRISPRa, serves as a tool to induce the over-expression of

genes and to examine its resulting effects (Konermann *et al*., 2015) [6]. Konermann *et al*.'s study used a genome scale CRISPRa screen to investigate the targeting rule of sgRNA. The primary components of the CRISPRa system include a sgRNA and a deactivated Cas9 protein (dCas9). The dCas9 protein, essentially a programmed Cas9 protein, retains its DNA-binding ability while losing its function of creating a double-strand break. Guided by the sgRNA, the whole dCas9 complex is directed to a particular locus, typically upstream of the target gene's promoter site, or the transcriptional starting site (TSS). One or more transcriptional activators are then recruited to the TSS under the guidance of sgRNA. These activators either directly or indirectly bind to the dCas9 protein, and interact with RNA polymerase or other transcriptional factors to stimulate RNA elongation or enhance RNA polymerase binding. This leads to an increase in transcription levels, thereby causing over-expression of gene. Multiple dCas9 complexes can be concurrently deployed to over-express several genes, thereby defining the relationship between a phenotype of interest and the expression level of certain genes.

2.2.1. CRISPRa Screen for Carcinogenesis

A notable study employed a CRISPRa screen for carcinogenesis to target a genome-scale range of genes within a liver injury model of mice (Wangensteen *et al*., 2018) [7].

Utilizing CRISPRa, researchers linked phenotypes with specific sgRNA targets locus in a vivo platform. In this liver injury model, researchers used CRISPRa to target a broad range of potential gene loci. Among these, the endogenous oncogene locus Myc was identified as a significant factor in carcinogenesis. By introducing sgRNAs targeting selected transcriptional start sites into mice liver cells and measuring sgRNAs enrichment, the researchers successfully identified the genes targeted by these sgRNAs. The result of the study presented the possibility of using CRISPRa in vivo for regulating the expression level of Myc.

2.2.2. CRISPRa Screen for Factors of Cellular Reprogramming

Researchers also have used CRISPRa to screen factors relevant to cellular reprogramming (Yang *et al*., 2019) [8]. Pluripotent stem cells (PSCs) have the capacity to divide and differentiate into an array of cell types that perform distinct functions. Consequently, they bear significant potential for medical application. Two PSC subtypes—ESCs (with a larger potential to differentiate) and EpiSCs (with a more limited differentiation scope)—have been found in mouse embryos. Previous studies revealed that over-expressing a select of few genes, such as Nanog, could reprogram EpiSCs into an ESC state. Taking the reprogramming of EpiSCs as their model to test CRISPRa, the researchers uncovered additional genes tied to this reprogramming process.

The study conducted a genome-scale CRISPRa with 87,863 sgRNAs to target over 19,994 upstream areas of genes. Ultimately, the gene Sall1emerged as a prime candidate, demonstrating a synergistic effect with Nanog during the reprogram-

ming process.

2.3. CRISPR Interference

CRISPRi, or CRISPR interference, serves as a newly developed method for gene repression. Unlike techniques that break the gene, CRISPRi deactivates the gene, thereby enabling a test for the effect of gene loss-of-function without disrupting the gene structure (Larson *et al.*, 2013) [9].

In this system, the primary components are a sgRNA and a dCas9 protein. The sgRNA directs the whole dCas9 complex to a particular locus, where the complex binds and consequently blocks the transcription process. The sgRNA can either lead the complex to the non-template strand of the exon, which blocks the elongation of transcription, or to the promoter region, which blocks the initiation of transcription. For eukaryotes, the dCas9 needs to be fused with a transcriptional repressor for efficient transcriptional blocking (Gilbert *et al.*, 2013) [10]. As the dCas9 binds to the DNA, RNA polymerase cannot bind or perform transcription, thereby silencing the gene. Notably, the gene can still be activated as dCas9 does not cleave the DNA strands. The deployment of multiple sgRNA and dCas9 complexes enables modulation of various genes, offering insights into the intricate pathways affecting diverse mechanisms.

2.3.1. CRISPRi Screen for Prostate Cancer

Researchers have used CRISPRi to screen for prostate cancer by targeting hundreds of risk SNP-containing CREs (rCREs) (Ahmed *et al.*, 2021) [11]. Prostate cancer (PCa) is associated with single nucleotide polymorphism (SNP) found in non-coding sequences. Prior studies have mapped over 160 SNP loci that elevate the PCa risk. Furthermore, SNPs have been found to reside in cis-regulatory elements (CREs), which regulate the transcription of neighboring genes. Building on these previous findings, the study focused on elucidating the function of these rCREs to identify pathway influencing cancer development. Utilizing CRISPRi screen, researchers targeted 270 PCa rCREs to examine how the loss of function of rCREs affected cancer expression. They found that one of the rCREs regulated the expression of the MYC oncogene, which was highly associated with PCa.

The researchers first engineered sgRNAs for each of the rCRE locus and transduced them into PCa cell lines. Over time, they monitored sgRNAs concentrations at various loci. A depletion of sgRNA over time signified the indispensability of each rCRE to cancer cell proliferation: the higher the depletion score, the larger the effect of the rCRE's knockout on cell growth. Notably, the researchers successfully identified multiple rCRE regions essential for cell proliferation specifically in PCa. Some of these rCRE, found to overlap in both cell lines, were identified as being located in the same genomic region, 8q24.21. Researchers found this region to be in high relevance with oncogenes MYC and PVT1. These rCREs were determined to promote cell proliferation by modulating MYC, thereby potentially fostering promoted cancer.

2.3.2. CRISPRi and CRISPRn Screens for Regulatory Elements Affecting Cell Proliferation

A study combined CRISPRi and CRISPRn techniques to explore regulatory elements that impact cell proliferation (Borys & Younger, 2020) [12]. In order to determine the function of non-coding regulatory elements, researchers selected the tumor repressor p53 as their model, given its known linkage with multiple enhancer elements.

To examine the downstream event of the p53 pathway, the team assembled a sgRNA library that targets thousands of p53 binding sites. They also included sgRNAs that specifically target p53 to validate the result. In the CRISPRn screen, researchers transected the chosen cell line with this sgRNA library. The team then utilized MAGeCK, a specialized computational tool for CRISPR analysis, to monitor changes in sgRNA abundance over time. They found that the sgRNAs targeting p53 were the most significantly enriched, which suggested that knocking out p53 led to increased cell proliferation. This result confirmed the validity of their screening approach. Further analysis identified several sgRNA-targeted p53-regulated genes, such as CDKN1A, whose knockouts similarly led to cell proliferation.

For the upstream analysis, the researchers designed a new sgRNAs library. This library targeted regulatory elements loci that had been previously identified in other studies. In the CRISPRi screen, researchers transduced both the dCAS9 protein and the new sgRNA library into the cells. Subsequent analysis with MAGeCK revealed that the most enriched sgRNAs targeted a specific location known as Peak974, which is situated upstream of the gene CDKN1A. This gene had already been implicated as promoting cell proliferation in the downstream pathway. Notably, the study implemented both CRISPRn and CRISPRi screening methods for each aspect of their research. However, they observed little congruence between the results obtained from the two methods. Ultimately, they concluded that CRISPRi offered a more accurate and reliable screening process, based on its alignment with the downstream pathway results.

3. Discussion

In conclusion, CRISPRn deactivates a gene while irreversibly breaking its DNA sequence; CRISPRi deactivates a gene in a reversible manner that allows it to be reactivated later. CRISPRa, in contrast, serves to activate genes. The strengths and weaknesses of the systems will be elaborated below.

The major difference between CRISPRn and CRISPRa/i is their reversibility, which is an advantage for CRISPRa/i but a major weakness for CRISPRn. CRISPRn, apart from CRISPRa and CRISPRi, uses a dCas9 which obtain a double-strand break irreversibly. Unlike CRISPRn, CRISPRa/i offers a reversible and more controllable method to manipulate gene expression and suppression. Thus, the off-target effect of CRISPRi/a is less significant than that of CRISPRn. While the dCas9 protein can unbind easily at any time, the off-target effect caused by CRISPRn's Cas9 protein is more severe as a double-strand break is more likely to cause an

undesired phenotype change, thus affecting the result of the screen. As shown in studies that used both CRISPRn and CRISPRi for screening regulatory elements, the latter technique yielded more accurate results. However, both technologies require Cas9 protein to reach the accurate transcription start site of its targeted area. Another limitation is that the irreversible nature of CRISPRn determines that multiple genes cannot be switched on and off to test how the combination of different genes creates a pathway for an interested phenotype. For both CRISPRn and CRISPRi, diseases caused by gene knockouts can be investigated, for example, cancer, as discussed in the case of lung metastasis.

The other major difference between the systems is their function on gene knockdown or over-expression. While CRISPRn and CRISPRi test the effect of gene knockout, CRISPRa holds an exclusive advantage: it is the only technology that can examine the phenotype when a certain gene is over-expressed, so it will be particularly helpful when discovering the genes related to diseases caused by gene over-expression. Common diseases caused by gene over-expression are: neurological disorders such as motor neuron disease caused by overexpression of the Human NF-H Gene (Meier *et al.*, 1999) [13]; and cancer, as discussed in this paper the effect of gene overexpression on carcinogenesis. When investigating the disease-causing genes of these above diseases, CRISPRa is a suitable choice.

The suitable applications of each technology show a coincidence: many diseases are caused by both gene knockouts and over-expression. Studies such as those exploring the screening for lung metastasis indicate that multiple genes contribute to complex genetic conditions like tumor growth. More importantly, findings reviewed in this paper relevant to cancer studies suggest that the multifaceted pathways of cancer, influenced by a variety of enhancers and repressors, make it clear that relying on a single screening technology may be insufficient for comprehensive analysis to identify genes associated with the phenotype. For instance, applying CRISPRa could identify disease enhancers but overlook repressors. Future work should therefore consider using a combination of CRISPRa and CRISPRi to yield a more holistic understanding of specific diseases.

4. Conclusions

My research highlights the extensive studies carried out using individual CRISPR technologies for single phenotype, and it underscores the need for more comprehensive research. Going forward, studies should look at the combined effects of gene knockouts and over-expression to unravel the complexity of mutations that may involve both repressors and promoters. Likewise, targeting both exons and introns can offer a more thorough understanding of a specific phenotype. For example, in CRISPRa screens focused on carcinogenesis, a combinatorial approach was used to identify both tumor enhancers and suppressors.

While genetic screening currently serves as a precursor to potential medical treatments by identifying the possible causes of diseases, the future holds promise for CRISPR's role in treatment itself. Post-genetic screening, CRISPRn could be used to edit genomes, enabling the removal or insertion of specific mutations.

Thus, the CRISPR system continues to hold tremendous potential not only for the understanding complex biological pathways but also for the potential cure of lethal diseases.

Conflicts of Interest

The author declares that they have no conflict of interest.

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