Application Of The CRISPR-Cas9 System to Drug Discovery in Breast Cancer

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Abstract. To elucidate the functioning of the CRISPR-Cas9 system in the realm of drug discovery, a series of experiments were undertaken. Firstly, CRISPR-Cas9 was employed to facilitate both positive and negative selection screening within human cells. Subsequently, an exploration of genes vital for pluripotent stem cell and cancer cell viability was conducted. Following this, in a melanoma model, a screen was performed to identify genes whose loss contributes to resistance against vemurafenib, a therapeutic agent targeting the mutant protein kinase BRAF. Notably, the top-ranking candidate genes included previously verified genes such as *NF1* and *MED12*, alongside novel discoveries in the form of *CUL3*, *NF2*, *TADA1*, and *TADA2B*. This investigation revealed a remarkable consistency among distinct guide RNAs directed at the same gene, with an elevated rate of successful hit confirmation. These findings underscore the potential of genome-scale screening employing Cas9, thus substantiating CRISPR-Cas9 as a potent methodology for large-scale sequencing and the selection of target sites.

Keywords: CRISPR-Cas9; drug discovery; breast cancer.

1. Introduction

Breast cancer is a prevalent and fatal disease affecting women globally. In developed nations, the 5-year survival rate can reach 80%, whereas in developing countries, it remains below 40% [1]. The formidable challenge before us lies in regulating and curing breast cancer. Conventional methods for treating breast cancer primarily involve chemotherapy and radiotherapy, which, though effective against cancer cells, inflict substantial harm on the body. In light of this, numerous researchers are striving to identify superior approaches for combatting breast cancer. Presently, the prevailing consensus underscores the critical importance of early detection in enhancing breast cancer outcomes and survival. Various drugs, such as raloxifene and tamoxifen, have proven effective in treating breast cancer [2]. Furthermore, a range of preventive measures, including targeted therapy, hormone therapy, and surgery, are employed in the battle against breast cancer. The burgeoning advancements in molecular biology have deepened our understanding of breast cancer and yielded more effective treatment methods.

The CRISPR-Cas9 system, initially discovered in bacteria, serves as a fundamental defense mechanism employed by bacteria to combat infections through precise nucleic acid base pairing, ultimately leading to the degradation of foreign genetic material. This technology enables the accurate modulation of gene expression by designing nucleic acid sequences. In this process, a short guide RNA binds to its target gene, forming a complex that subsequently facilitates the enzymatic cutting of the target gene, resulting in its knockout and the silencing of its expression. The CRISPR system can be categorized into two types: pooled and arrayed. Pooled CRISPR offers scalability advantages, making it suitable for drug discovery [3]. In general, the mechanism of drug discovery using CRISPR/Cas involves the identification of potential key genes responsible for anti-tumor immunity through CRISPR library screening. In 2021, Wang et al. demonstrated that knocking out the E3 ubiquitin ligase Cop1 using the CRISPR system enhances anti-tumor immunity, suggesting Cop1 may be a key or essential gene and a potential target for drug discovery [4]. In the pursuit of new drug discovery, the interaction between drugs and targets plays a pivotal role. Target validation, which involves introducing resistance-inducing mutations into wild-type cells, is a crucial step. Combining CRISPR-Cas9 technology with whole-genome sequencing and drug resistance mutation

screening can expedite target validation and the discovery of novel drug targets, exemplified by the discovery of rapamycinTOR1 [5]. In 2015, the precise introduction of XPO1C528S point mutation into “Jurkat” acute T-cell leukemia cells using CRISPR-Cas9 revealed that selinexor (PKT-330) targets and blocks the nuclear export protein receptor XPO1 [6].

CRISPR-Cas9 technology has also found application in the treatment of breast cancer, with numerous studies exploring its potential in this context and this versatility positions it as a promising avenue for breast cancer treatment [7]. One application of CRISPR-Cas9 in breast cancer treatment involves immunotherapy.

Though CRISPR-Cas9 technology has evolved into one of the most effective gene-targeted editing tools, it does have certain limitations, such as a certain rate of off-target effects. Many scholars are actively addressing this issue and have made significant progress. It is essential to recognize that CRISPR has the potential to address epigenetic modifications, another factor implicated in breast cancer development alongside genetic variations. CRISPR-Cas9, with its high specificity, offers a promising avenue for targeting specific forms of breast cancer. Further research employing CRISPR-Cas9 technology in the study of breast cancer promises to provide new experimental evidence and theoretical insights, ultimately advancing the development of novel and effective treatments.

2. Methods

2.1. Data Source

In this article, the research is divided into three parts. The data used in the first part, which can also be referred to as the "drug target site selection," is derived from the article published by Ophir Shalem et al. in Science in 2014. The data used in the second part, which can be termed "gene repair," is derived from the article published by Yuxuan et al. in Cell Stem Cell in 2013. The data used in the third part, relating to "precision medicine and personalized medicine development," is derived from the article published by Norah et al. in Nature in 2017.

2.2. Index Selection and Description

In the first part, the selected indices and their descriptions include "drug resistance of melanoma cells" and the "target-missing rate." In the second part, the primary indices and descriptions focus on "NHEJ-mediated repair/non-repair" and "HDR-mediated repair." In the third part, the main indices and descriptions revolve around "phenotypic characterization," "assessment of putative off-target and on-target mutations," and "transcriptome analysis."

2.3. Method Introduction

Firstly, a substantial sgRNA library containing numerous genes, with each gene designed to have 3-4 sgRNAs, was constructed. It is worth noting that these sgRNAs have been verified to exhibit the lowest target-missing rate. Lentiviruses were utilized as delivery vectors, and a lentiviral CRISPR library containing plasmids was synthesized. These plasmids were transfected into in vitro cells, and the occurrence of gene editing functions was subsequently assessed. If gene editing functions were observed, the target genes were then analyzed. These newly identified target genes may serve as potential drug target sites.

Secondly, a mouse model exhibiting a dominant cataract disease due to a well-defined mutation in Crygc (1-bp deletion in Crygc exon 3) was deliberately selected for this study. Using this model, five distinct sgRNAs were designed, each targeting various regions within the mutant Crygc gene. Specifically, sgRNA-1/-2/-3 was directed at regions encompassing the site of the 1-bp deletion within the mutant allele. On the other hand, sgRNA-4 was tailored to target the region downstream of the 1 bp deletion, while sgRNA-5 aimed to address the normal sequences upstream of the deletion. To evaluate the feasibility of CRISPR-Cas9-regulated gene repair, initial assessments were performed at the cellular level. Subsequently, investigations were conducted to determine if the genetic anomaly within the cataract mouse model was rectified at the organismal level through the introduction of

CRISPR-Cas9 into zygotes. Furthermore, a supplementary experiment was carried out to assess whether the provision of an exogenous WT single-stranded DNA oligo, denoted as 'Oligo-1,' could enhance the efficacy of HDR-regulated genetic repair. This enhancement was pursued by co-injecting Oligo-1, sgRNA-4, and Cas9 mRNA into the cytoplasms of zygotes carrying the heterozygous cataract mutation.

Thirdly, with the patient's consent, a human embryo was obtained. CRISPR-Cas9 was employed for genome editing to investigate the function of OCT4 during human embryogenesis. OCT4-targeting guide RNA was used, and various aspects were examined, including the transcriptome, orthologous gene expression, and blastocyst development in human embryo cells.

3. Results and discussion

3.1. The Use of CRISPR-Cas9 in New Drug Target Sites Selected

The RNA-guided CRISPR-Cas9 system proved to be a promising technique for inducing precise genetic alterations at specific genomic loci. Lentiviral vectors are frequently employed for delivering pooled shRNAs. This choice is due to their ease of titration for controlling transgene copy numbers and their stable maintenance as genomic insertions during subsequent cell division. Consequently, we devised a single lentiviral vector, denoted as "lentiCRISPR," to facilitate the delivery of a puromycin selection marker, sgRNA, and Cas9 into target cells, as illustrated in Figure 1A. This distinctive feature of delivering both sgRNA and Cas9 concurrently via a single vector extends its applicability to a wide range of cell types without the prerequisite of establishing Cas9-expressing cell lines in advance. Subsequent to transduction at a low multiplicity of infection (MOI=0.3), and subsequent puromycin selection, lentiCRISPRs effectively extinguished EGFP fluorescence in 93±8% (mean±SD) of cells within 11 days, as depicted in Figure 1B. Conversely, when cells were transduced with lentiviral vectors carrying EGFP-targeting shRNAs, this resulted in an incomplete silencing of EGFP fluorescence, as shown in Figure 1C.

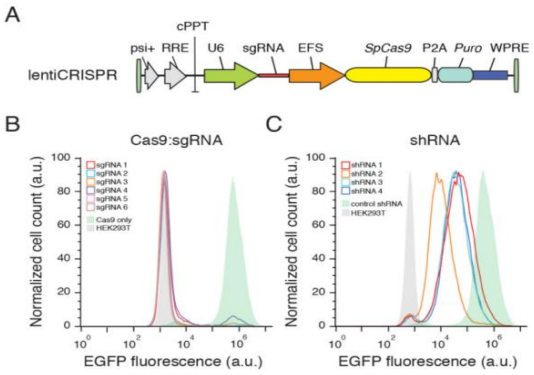

Fig. 1 Lentiviral delivery of sgRNA and Cas9 offers effective depletion of target genes [8]

A similar screen was conducted to identify protein kinase inhibitor vemurafenib (PLX) drug resistance using a comprehensive pool containing 90,000 shRNAs. Firstly, we computed the cumulative P-value distribution for the top 100 hits utilizing RIGER scoring, as illustrated in Figure 2A. In this context, lower P-values in the GeCKO vs shRNA screen demonstrate a more consistent scoring pattern among sgRNAs. Subsequently, in the assessment of the top 10 RIGER-hit genes, we found that 78±27% of sgRNAs targeting each gene were ranked within the top 5% of enriched sgRNAs, in contrast to a mere 20±12% of shRNAs targeting the same genes, as depicted in Figure 2B. In the case of NF2, 4 out of 5 sgRNAs led to >98% allele modification just 1 week following transduction, and all 5 sgRNAs exhibited >99% allele modification 2 weeks post-transduction, as displayed in Figure 2C. We conducted a comparative analysis of protein depletion and PLX resistance achieved through SgRNA and shRNA-mediated methods. This involved Western blot analysis, shown in Figure 2D, as well as cell growth assays, displayed in Figure 2E. Intriguingly, although all

sgRNAs could confer resistance to PLX, only the most effective shRNA attained a substantial reduction in sensitivity to PLX (Figure 2E). This suggests that even minimal levels of NF2 are adequate to maintain sensitivity to PLX.

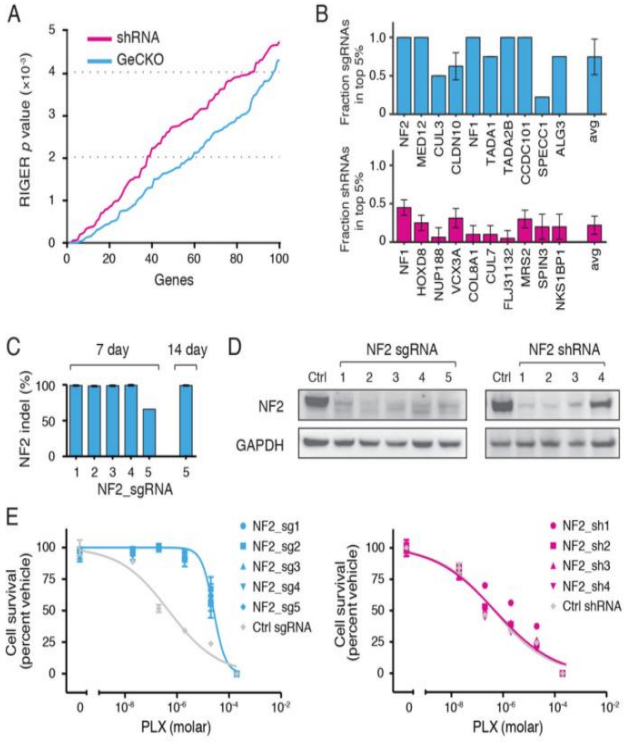

Fig. 2 Comparison of shRNA and GeCKO screens and verification of NF2 [8]

In recent years, some countries have continued to strengthen their investment in drug discovery. However, the ability to discover new drugs still lacks innovation. For more than half a century, the field has been gradually moving towards a new level of original innovation segment transformation. New drug discovery faces new challenges, so researchers should adjust their research directions accordingly.

3.2. The Use of CRISPR-Cas9 in Gene Repaired

sgRNA-4 and Cas9 mRNA were concurrently introduced into the cytoplasms of zygotes. These injected zygotes successfully matured into blastocysts at an impressive rate of 91%, as evidenced by the data provided in Table 1, signifying minimal toxicity associated with the administered RNAs. Among the 135 transferred blastocysts (TBs) generated following the injection, 22 healthy live pups were subsequently born, as detailed in Table 1. The nonhomologous end joining (NHEJ)-mediated and homology-directed repair (HDR) rates using this method were 2% and 4%, respectively.

Table 1. Gene repair in cataract mice facilitated by CRISPR-Cas9 technology [9]

Oligo	Injected embryos	Blastocysts (Percentage of injected embryos)	TBs	Live-born pups	Genetic modification		NHEJ-mediated repair/non-repair	HDR-mediated repair
					WT allele	Mutant allele		
-	172	157(91%)	135	22	0	10	2/4	4
Oligo-1	245	213(87%)	178	29	0	14	4/5	5
Oligo-2	221	190(86%)	159	27	0	12	5/3	4

After infecting Oligo-1, 29 live pups were born from 178 TBs (Table 1). The NHEJ-mediated and HDR rates using this method were 4 and 5, respectively.

Following the infection of Oligo-2, 27 live pups were born from 159 TBs (Table 1). The NHEJ-mediated and HDRrates using this method were 5 and 4, respectively.

In conclusion, these results indicate that although the provision of exogenous oligos may not be obligatory, exogenous oligonucleotides can be utilized as an alternative template for mending the mutated gene, particularly when the native allele cannot be employed as template.

3.3. The Use of CRISPR-Cas9 in Precision Medicine

Precision medicine and personalized medicine development involve utilizing genetic and representational information from individuals to formulate a tailored treatment plan. It encompasses four key aspects: precision, timeliness, sharing, and personalization. The CRISPR-Cas9 system, a recent development, offers a novel approach for precise genome modification. It has been employed to assess the role of OCT4 during human embryogenesis. The absence of the OCT4 phenotype was detected in 54% of embryos that received injections of Cas9 mRNA and sgRNA2b, in contrast to 0%, 10%, or 3% for embryos that were injected with Cas9 mRNA along with sgRNA1-1, sgRNA1-2, or sgRNA4, respectively (Figure 3). These results indicate that outperforms the other tested sgRNAs in eliciting null mutations in both human ES cells and mouse embryos. These findings underscore the significant differences among individuals, and we can thus speculate that CRISPR-Cas9-modulated genome editing is a potent approach for studying gene functions during human development. This holds particular promise in the fields of precision medicine and personalized medicine, where treatments must be tailored to the unique genetic profiles of different individuals.

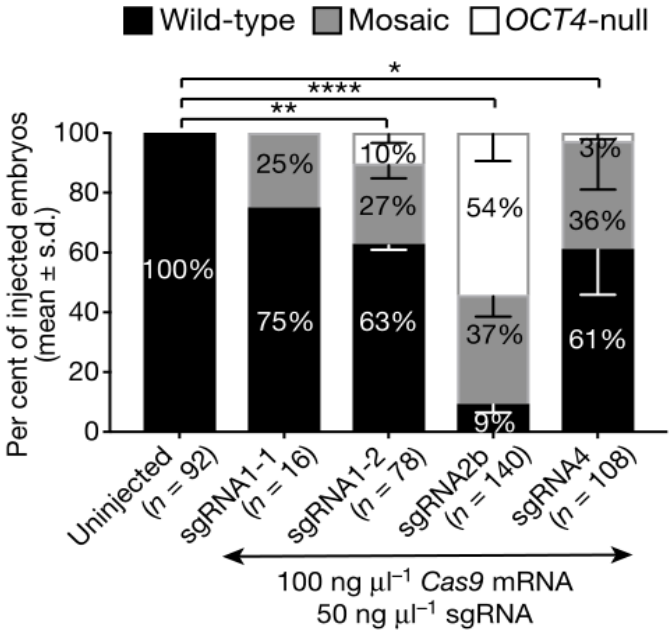

Fig. 3 Screening of OCT4-targeted sgRNAs in CRISPR–Cas9 knockout mouse embryos and human ES cells [10]

3.4. The Brief Evolution of CRISPR-Cas9

Figure 4 illustrates the concise progression of CRISPR-Cas9, from its discovery to its application in breast cancer research. It is evident that CRISPR-Cas9 ison the verge of emerging as a formidable tool in the field of medicine.

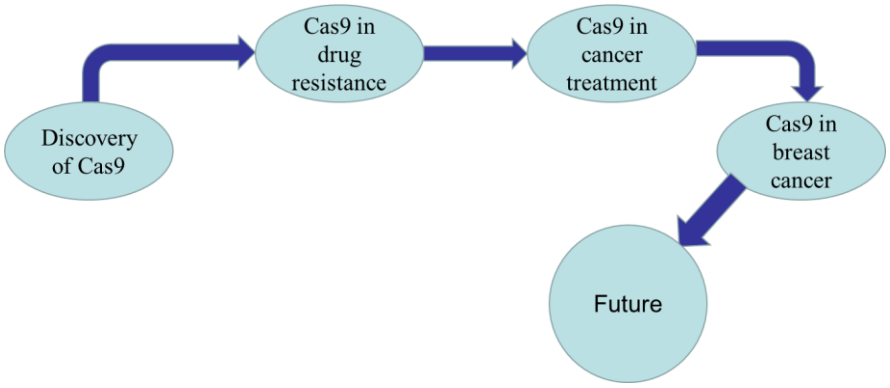

Fig. 4 The use progress of CRISPR-Cas9

4. Conclusion

This article reviewed the applicability of the CRISPR-Cas9 system in the selection of new drug target sites, gene repair, and its role in precision medicine and personalized medicine development. Through a series of studies, researchers have amassed a wealth of detailed data. These findings further support the role of the CRISPR-Cas9 system in cancer therapy. Based on previous research, there is a consensus that CRISPR-Cas9 is an effective method for genome-scale sequencing and target site selection, a crucial aspect of identifying new drug targets. Furthermore, this study provides compelling evidence for the potential use of the CRISPR-Cas9 system in rectifying genetic diseases, offering a promising avenue for mitigating genetic disorders.

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