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Optimising CRISPR-Cas9 for Targeted Cancer Therapy: Techniques and Challenges

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Abstract

Globally, cancer is the second most common cause of mortality and continues to present significant social and economic challenges. Despite the fact that we are gaining a deeper understanding of the molecular causes of cancer, there is still a need for additional research to be conducted in order to create new therapeutic approaches and technologies that leverage these discoveries. The CRISPR-Cas9 genome editing technique has gained recognition in the field of cancer treatment due to its exceptional efficiency and accuracy. In numerous disciplines, including the investigation of chemical-genetic interactions and the identification of novel cancer treatment targets, CRISPR-Cas9 has demonstrated remarkable therapeutic potential. This technology offers critical new insights into the manner in which malignancies respond to medication treatment. Additionally, CRISPR-Cas9 can be employed in cancer immunotherapy to rapidly modify immune cells and oncolytic viruses. CRISPR-Cas9's most critical attribute is its precise gene editing capabilities, which extend beyond human tissue and cell culture models and can be employed for therapeutic research. This study examines critical factors that must be taken into account when using CRISPR/Cas9 in therapeutic applications, as well as significant obstacles that must be overcome prior to its clinical application in the treatment of a genetically influenced and intricate disease such as cancer.

Keywords: CRISPR-Cas9, cancer, genome editing, immunotherapy, pharmacological therapy

Introduction

The discovery of regularly spaced short palindromic repeats (CRISPR) [1, 2, 3] and their function as a responsive prokaryotic immune system when combined with CRISPR-associated (Cas) genes [4, 5, 6, 7] have made it possible to use CRISPR as a powerful genome-engineering tool [8,9,10, 11]. It is commonly acknowledged that the most significant advancement in biotechnology of the twenty-first century is the CRISPR/Cas9 system. This is because of its unique blend of efficacy and specificity, together with its comparatively simple handling. Furthermore, it has opened up new avenues for precise genetic material alteration and visualisation inside living things. All things considered, CRISPR/Cas9 has proven to have an unparalleled therapeutic potential. This capability has made it possible to investigate diseases and treat them precisely, and it has also opened the door for the creation of novel drugs. Arguably, its most important potential is that it holds the promise of novel approaches to diagnosis and treatment.

Literature Review

Utilizing consistently spaced short palindromic repeats (CRISPR) as a potent genome-engineering tool has been made possible by the discovery of CRISPR [1, 2, 3] and its ability to function as an adaptable prokaryotic immune system when paired with CRISPR-associated (Cas) genes [4,5, 6, 7]. The CRISPR/Cas9 system is widely regarded as the most important development in biotechnology of this century. This is due to its distinct combination of selectivity and efficacy as well as its relatively easy handling. It has also created new opportunities for the exact manipulation and viewing of genetic material inside living organisms. Taking everything into account, CRISPR/Cas9 has shown to have therapeutic potential that is unmatched. This skill has paved the way for the development of innovative medications and allowed for the exact investigation and treatment of illnesses. Its potential for innovative methods of diagnosis and treatment is perhaps its greatest asset.

CRISPR/Cas9 delivery

There are three different kinds of CRISPR/Cas systems, each of which has numerous subgroups, according to the commonly accepted taxonomy [13]. The three parts of the type II CRISPR/Cas system are as follows. The parts are an endonuclease called Cas9, a transactivating crRNA called tracrRNA, and a CRISPR RNA called crRNA. The most widely used gene editing method is the type II CRISPR/Cas system [8]. The crRNA and tracrRNA molecules unite to form the structure once the guide RNA (gRNA) creates a duplex structure. A synthetic fused chimeric single gRNA (sgRNA) can be employed in place of this structure, simplifying the application of CRISPR/Cas9 in the field of genome engineering [8]. The target DNA location is designed primarily for this purpose and is expected to contain a complementary sequence of twenty base pairs (bp). This

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is followed by a brief region of DNA known as the "protospacer-adjacent motif" (PAM). It is necessary to assemble this sequence in the correct order. This sequence is essential to guarantee compatibility with the Cas9 protein that is being used. After the synthesis of the Cas9 nuclease and messenger RNA (sgRNA) in the cell, they will combine to form a ribonucleoprotein (RNP) complex. This will take place after messenger RNA is synthesised. This complex will be directed to a particular location on the DNA by the sgRNA.

Short guide RNA (sgRNA) is attached to the target region by Watson-Crick base-pairing after Cas9 accurately cleaves the DNA to produce a double-strand break (DSB). The blunt ends are produced by a cleavage that takes place in the protospacer, exactly three nucleotides upstream of the PAM. The RuvC and HNH active-site motifs present in Cas9 aid in the cleavage of antiparallel DNA strands. These motifs may affect the positive (+) and negative (-) strands, respectively, according to references [14, 15]. This double-strand break (DSB) is repaired by the cell's machinery through the use of non-homologous end joining (NHEJ) or homology-directed repair (HDR) techniques [16, 17, 18]. These two methods are both frequently referred to as supplementary healing mechanisms. The cell's state and the accessibility of a repair template determine the best course of action. By recombining donor DNA at the location of the double-strand break, the HDR mechanism accomplishes precise repair. Homology-directed repair is a valuable strategy because it may be used to insert particular sequences or mutations into a targeted section of the genome. We are interested in a specific region of the genome. The most common method, known as NHEJ, creates indels by introducing haphazard nucleotide insertions or deletions at the site of a double-strand break (DSB). Errors are more likely to be made when using this approach. As such, it can be used to generate frameshift mutations that result in targeted gene knockouts (KO) (Figure 1).

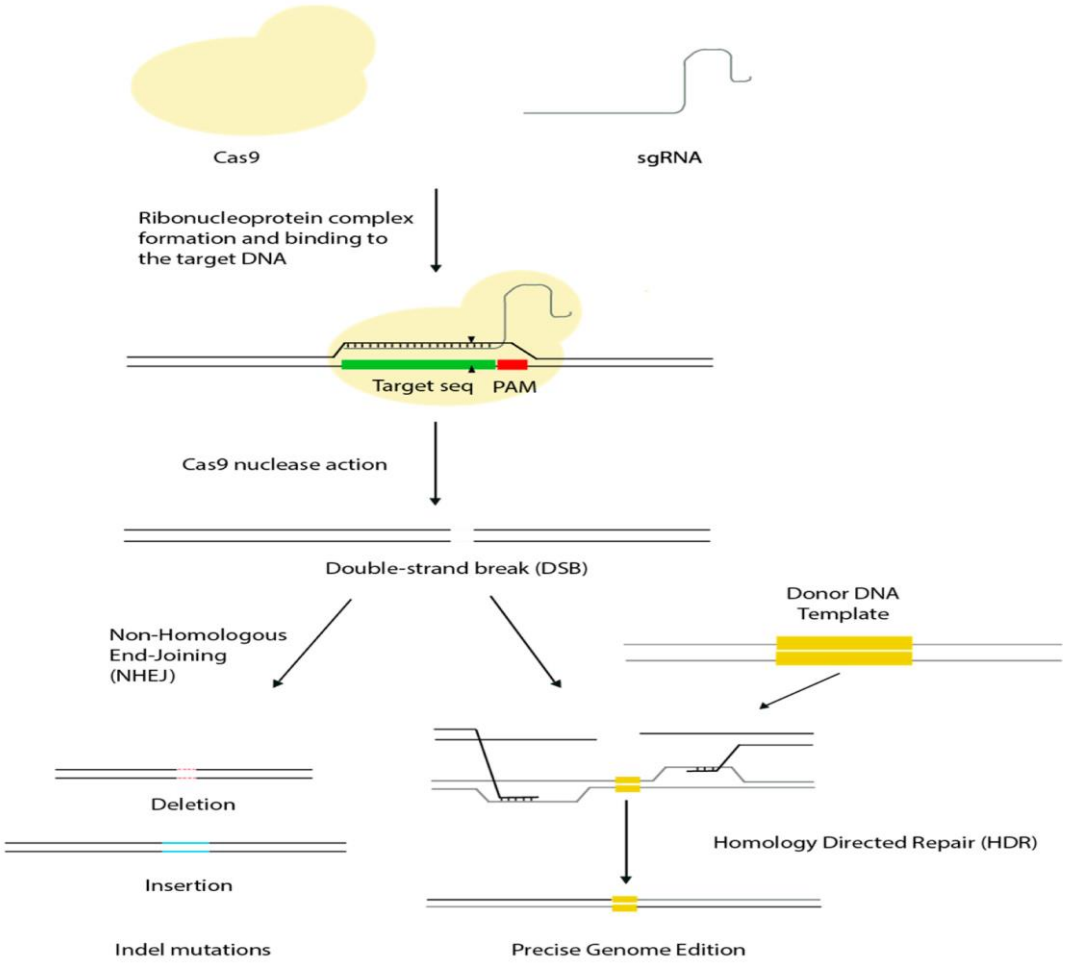

Figure 1: ***How CRISPR/Cas9 works. Cas9 and sgRNA produce ribonucleoprotein complexes. This complex cuts double-stranded DNA (black triangles) by annealing with base-pairing complementarity and detecting the protospacer-adjacent motif (PAM) sequence next to the genomic target sequence. NHEJ or HDR pathways begin with double-strand breaks. When there is no homologous repair template, NHEJ inserts random base pair indels. The homology-directed repair process and donor DNA template enable accurate genome editions.***

Process and advantages of CRISPR genomic editing

With the use of Cas9 and clustered, regularly interspaced short palindromic repeats (CRISPR), this method allows for the accurate and efficient cleavage of a particular target DNA sequence. Moreover, this method has made the synthesis of small guide RNAs (sgRNAs) much more straightforward, which makes genome editing easier. With the use of several different sgRNAs, multiplexing is a possibility with this strategy. This tactic offers an extra advantage. The only nuclease that can edit several loci at once is the CRISPR/Cas9 system [19,20, 21]. Short guide RNAs that target different places particularly are inserted to accomplish this. This particular feature sets this group of nucleases apart from others that are utilised in genome editing. In fact, introducing two short guide RNAs into a single cell can result in small deletions [10], complex rearrangements [22, 23], and even chromosomal inhibition [24]. The outcomes could range from straightforward reorganisations to minor removals. Another essential feature of this structure is the adaptability of the CRISPR/Cas9 system. The CRISPR/Cas9 components and interactors have been improved and adjusted, which has not only improved the system's accuracy and efficiency but also broadened its potential applications beyond editing [25].

Enhancing the CRISPR technology's DNA specificity has been a top priority in the research because of the high rate of off-target activity, which has been supported by multiple studies [26]. This has happened because off-target behaviours have been confirmed. As a result, many strategies have been developed to lower the quantity of unsaleable goods. A potential approach could be to switch from plasmid distribution to direct cellular delivery of laboratory-made RNP complexes. With this method, Cas9 and sgRNA expression is more durable and stable over a more extended period. Moreover, it results in very efficient editing by improving the ratio of targeted genome editing to non-targeted editing in mammalian cells [27,28,29,30,31]. The use of Cas9 variants that have been altered to respond to light or small molecules [32, 33, 34], split Cas9 variants for controlled reassembly and allosterically regulated Cas9 [35, 36, 37] are further options that can be investigated. After Cas9 was redesigned to create nicks in a single strand of DNA, using two Cas9 nickases was possible. This was made more accessible by the change. These nickases are directed by two different gRNAs that target the same locus even though they are located on different DNA strands. This method reduces off-target events while producing very accurate DNA cleavage with an efficiency close to that of conventional CRISPR/Cas9. 38 is the value. A similar strategy to the one outlined above uses two catalytically inactive Cas9 mutants coupled to the FokI nuclease (fCas9) so that the FokI nuclease is only functional when it is dimerised. The fCas9 nucleases can successfully alter a target region since they have been shown in human cells to exhibit selectivity greater than 140 times that of the wild-type Cas9 nuclease. Numerous different methods have confirmed this. In summary, research on the mutational analysis of Cas9 has demonstrated that the unwanted electrostatic interactions between Cas9 and its target DNA can be eliminated by introducing three to four particular point mutations. As a result, Cas9's activity's specificity is significantly improved [40,41].

While there are certain benefits to CRISPR/Cas9 technology over conventional programmable nucleases for genome editing, there are also some drawbacks that should be taken into account. CRISPR/Cas9 technology is still in its early stages of development; thus, improvements are needed to increase its efficiency and its capacity to target particular sequences. It is anticipated that these improvements will become essential in the future. The creation of a safe, efficient, and cell-specific delivery mechanism for CRISPR/Cas9 is still a significant challenge, and there is also a need to lessen the effects of unintentional effects.

Drug Target Discovery CRISPR/Cas9 Library Screens

The identification and functional analysis of unknown genes is a typical application of high-throughput genomic screening technology. Numerous critical biological pathways and signalling networks have been successfully identified via mutagenesis screening. Using this method, it is possible to identify the genes that cause a particular phenotype. However, the creation of heterozygous mutants as a result of applying unknown random mutations is a significant drawback of using mutagenesis screens for target drug development. Targeted RNA interference (RNAi), one of several possible strategies, can be used to get around this restriction. Despite the fact that high-throughput RNAi genomic library screens have provided insightful information about the connections between particular genes and symptoms associated with loss of function, there are still a number of unanswered questions. This strategy has two shortcomings: noticeable off-target effects and inefficient knockdown, sometimes known as incomplete knockdown. 43 is the value. In addition, there are several advantages to using CRISPR/Cas9 as opposed to RNA interference. This method's ability to target every region of the genome, including intergenic regions, enhancers, promoters, and introns, is one of its advantages [44]. Several benefits are at your disposal, including the capacity to target the entire genome, outstanding repeatability, and total inactivation (sometimes referred to as total knockdown). Recent years have seen a rapid creation of CRISPR/Cas9 libraries thanks to advancements in functional RNAi platforms. This accomplishment

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has been made possible by the knowledge that was gained. In 2013, it was asserted, based on two different articles [44, 45], that CRISPR libraries were more successful than RNA interference libraries.

As of right now, there are three main uses for genome-wide CRISPR libraries: (1) CRISPR-based loss-of-function (CRISPR knock-out), which is used to find new biological mechanisms such as drug resistance and cell survival signals [45], (2) CRISPR-based gene activation (CRISPRa), which helps find gain of function, [46], and (3) CRISPR-based gene inhibition (CRISPRi), which finds use in finding loss of function. CRISPRa and CRISPRi libraries use catalytically degraded Cas9 (dCas9) in conjunction with regulatory cofactors such VP64 (activation) [48] or the Krüppel associated box (KRAB) repression (inhibition) [49], whereas CRISPR knock-out libraries typically use unmodified Cas9. To improve CRISPRa activity, other components have been created, such as VP64-p65-Rta (VPR), Synergistic Activation Mediators (SAM), or SunTag [50,51] (Figure 2).

Because CRISPR is flexible, screens for positive and negative selection can be carried out. Positive selection screens are used to identify genes that improve cell survival in particular circumstances, such as those enforced by a drug therapy. This is demonstrated by the application of CRISPR library therapy on cells, which is followed by the introduction of a medication that fights cancer in the affected cells. Only gRNAs that have endured and are drug-resistant can be extracted for analysis in order to conduct the study [52]. It is possible to identify potential genes linked to drug resistance by using the gRNA sequences. On the other hand, under specific conditions, unfavourable selection is used to efficiently identify cells that are either dead or growing poorly. This is accomplished through the process of selection; with the use of this technology, it is possible to identify genes that are essential for survival and that may serve as valuable targets for molecular-level treatments. For example, if a pool of gRNAs is used to generate a set of randomly selected mutants, the cells containing gRNAs that precisely target a survival-critical gene would not be able to endure. This happens because the gRNAs target the gene specifically. Only the cells that have specifically targeted non-essential genes for the survival of the organism would remain after a predetermined number of cycles. With next-generation sequencing, it is possible to sequence a set of gRNAs from the initial state and the surviving state in order to determine which genes are essential for survival. The application of next-generation sequencing makes this feasible.

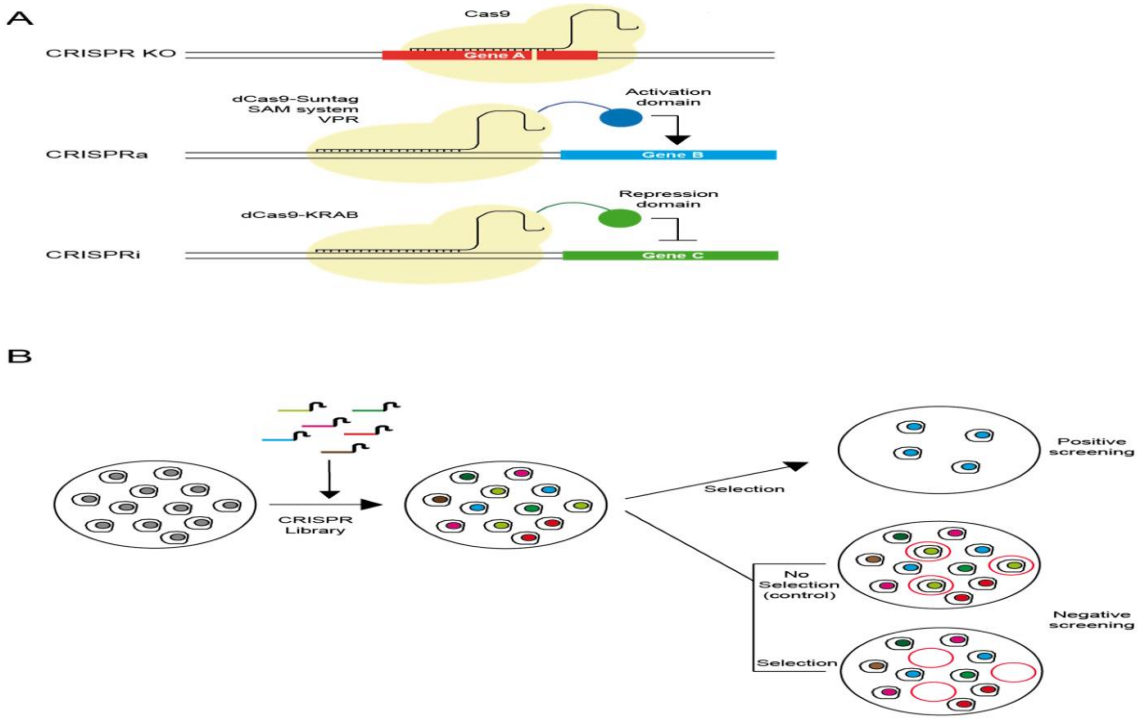

Figure 2: ***Main uses of CRISPR in the search for new drugs. (A) CRISPR KO, which uses the wild-type CRISPR system to produce non-functional proteins or knock out genes; (ii) CRISPR activation, which uses a catalytically inactive version of the Cas9 enzyme (dCas9) fused to different activator domains (like SunTag, SAM, and VPR) to activate specific genes; and (iii) CRISPR repression or inactivation are the three main techniques for modulating transcription. In pooled high-throughput screening, genome-scale guide RNA libraries can be selected using positive or negative criteria.***

Conclusion

The fact that CRISPR-mediated gene editing has already been shown to have therapeutic potential indicates how promising this technique is. However, it will be essential to make sure that this technology is delivered into target tissues in a way that is both safe and effective if it is to be successfully used in clinical settings. The high expectations surrounding CRISPR gene editing must be combined with strategic planning to ensure the successful growth of this cutting-edge gene editing-based modality. Enabling regulatory structures should be put in place as part of this planning. Despite this, it is clear that further optimisation of the technology is required before it can be widely used in clinical settings, especially in relation to safety, effectiveness, and specificity. Even if there are still some challenges to be solved, we anticipate that the continued development of this gene-editing technique will improve the cancer medicines that are now on the market.

References

1. Ishino, Y.; Shinagawa, H.; Makino, K.; Amemura, M.; Nakata, A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. J. Bacteriol. 1987, 169, 5429–5433.
2. Mojica, F.J.; Juez, G.; Rodríguez-Valera, F. Transcription at different salinities of Haloferax mediterranei sequences adjacent to partially modified PstI sites. Mol. Microbiol. 1993, 9, 613–621.
3. Van Soolingen, D.; de Haas, P.E.; Hermans, P.W.; Groenen, P.M.; van Embden, J.D. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of Mycobacterium tuberculosis. J. Clin. Microbiol. 1993, 31, 1987–1995.
4. Bolotin, A.; Quinquis, B.; Sorokin, A.; Ehrlich, S.D. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology 2005, 151, 2551–2561.
5. Mojica, F.J.M.; Díez-Villaseñor, C.; García-Martínez, J.; Soria, E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J. Mol. Evol. 2005, 60, 174–182.
6. Pourcel, C.; Salvignol, G.; Vergnaud, G. CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. Microbiology 2005, 151, 653–663.
7. Van der Oost, J.; Jore, M.M.; Westra, E.R.; Lundgren, M.; Brouns, S.J.J. CRISPR-based adaptive and heritable immunity in prokaryotes. Trends Biochem. Sci. 2009, 34, 401–407.
8. Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J.A.; Charpentier, E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 2012, 337, 816–821.
9. Cho, S.W.; Kim, S.; Kim, J.M.; Kim, J.-S. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat. Biotechnol. 2013, 31, 230–232.
10. Cong, L.; Ran, F.A.; Cox, D.; Lin, S.; Barretto, R.; Habib, N.; Hsu, P.D.; Wu, X.; Jiang, W.; Marraffini, L.A.; et al. Multiplex genome engineering using CRISPR/Cas systems. Science 2013, 339, 819–823.
11. Mali, P.; Esvelt, K.M.; Church, G.M. Cas9 as a versatile tool for engineering biology. Nat. Methods 2013, 10, 957–963.
12. Barrangou, R.; Fremaux, C.; Deveau, H.; Richards, M.; Boyaval, P.; Moineau, S.; Romero, D.A.; Horvath, P. CRISPR provides acquired resistance against viruses in prokaryotes. Science 2007, 315, 1709–1712.
13. Makarova, K.S.; Haft, D.H.; Barrangou, R.; Brouns, S.J.J.; Charpentier, E.; Horvath, P.; Moineau, S.; Mojica, F.J.M.; Wolf, Y.I.; Yakunin, A.F.; et al. Evolution and classification of the CRISPR-Cas systems. Nat. Rev. Microbiol. 2011, 9, 467–477.
14. Gasiunas, G.; Barrangou, R.; Horvath, P.; Siksnys, V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proc. Natl. Acad. Sci. USA 2012, 109, E2579–E2586.
15. Mojica, F.J.M.; Díez-Villaseñor, C.; García-Martínez, J.; Almendros, C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. Microbiology 2009, 155, 733–740.
16. Liang, F.; Han, M.; Romanienko, P.J.; Jasin, M. Homology-directed repair is a major double-strand break repair pathway in mammalian cells. Proc. Natl. Acad. Sci. USA 1998, 95, 5172–5177.
17. Bibikova, M.; Carroll, D.; Segal, D.J.; Trautman, J.K.; Smith, J.; Kim, Y.G.; Chandrasegaran, S. Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. Mol. Cell. Biol. 2001, 21, 289–297.
18. Fishman-Lobell, J.; Haber, J.E. Removal of nonhomologous DNA ends in double-strand break recombination: The role of the yeast ultraviolet repair gene RAD1. Science 1992, 258, 480–484.
19. Jakočiūnas, T.; Bonde, I.; Herrgård, M.; Harrison, S.J.; Kristensen, M.; Pedersen, L.E.; Jensen, M.K.; Keasling, J.D. Multiplex metabolic pathway engineering using CRISPR/Cas9 in Saccharomyces cerevisiae. Metab. Eng. 2015, 28, 213–222.

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Vol. 28 Iss. 3 (2024)

20. Wang, H.; Yang, H.; Shivalila, C.S.; Dawlaty, M.M.; Cheng, A.W.; Zhang, F.; Jaenisch, R. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 2013, 153, 910–918.
21. Li, W.; Teng, F.; Li, T.; Zhou, Q. Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. Nat. Biotechnol. 2013, 31, 684–686.
22. Torres, R.; Martin, M.C.; Garcia, A.; Cigudosa, J.C.; Ramirez, J.C.; Rodriguez-Perales, S. Engineering human tumour-associated chromosomal translocations with the RNA-guided CRISPR-Cas9 system. Nat. Commun. 2014, 5, 3964.
23. Choi, P.S.; Meyerson, M. Targeted genomic rearrangements using CRISPR/Cas technology. Nat. Commun. 2014, 5, 3728.
24. Adikusuma, F.; Williams, N.; Grutzner, F.; Hughes, J.; Thomas, P. Targeted Deletion of an Entire Chromosome Using CRISPR/Cas9. Mol. Ther. 2017, 25, 1736–1738.
25. Dominguez, A.A.; Lim, W.A.; Qi, L.S. Beyond editing: Repurposing CRISPR-Cas9 for precision genome regulation and interrogation. Nat. Rev. Mol. Cell Biol. 2016, 17, 5–15.
26. Tsai, S.Q.; Joung, J.K. Defining and improving the genome-wide specificities of CRISPR-Cas9 nucleases. Nat. Rev. Genet. 2016, 17, 300–312.
27. Kim, S.; Kim, D.; Cho, S.W.; Kim, J.; Kim, J.-S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res. 2014, 24, 1012–1019.
28. Lin, S.; Staahl, B.T.; Alla, R.K.; Doudna, J.A. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. Elife 2014, 3, e04766.
29. Ramakrishna, S.; Kwaku Dad, A.-B.; Beloor, J.; Gopalappa, R.; Lee, S.-K.; Kim, H. Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. Genome Res. 2014, 24, 1020–1027.
30. Zuris, J.A.; Thompson, D.B.; Shu, Y.; Guilinger, J.P.; Bessen, J.L.; Hu, J.H.; Maeder, M.L.; Joung, J.K.; Chen, Z.-Y.; Liu, D.R. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nat. Biotechnol. 2015, 33, 73–80.
31. Torres-Ruiz, R.; Martinez-Lage, M.; Martin, M.C.; Garcia, A.; Bueno, C.; Castaño, J.; Ramirez, J.C.; Menendez, P.; Cigudosa, J.C.; Rodriguez-Perales, S. Efficient Recreation of t(11;22) EWSR1-FLI1+ in Human Stem Cells Using CRISPR/Cas9. Stem Cell Rep. 2017, 8, 1408–1420.
32. Nihongaki, Y.; Kawano, F.; Nakajima, T.; Sato, M. Photoactivatable CRISPR-Cas9 for optogenetic genome editing. Nat. Biotechnol. 2015, 33, 755–760.
33. Dow, L.E.; Fisher, J.; O'Rourke, K.P.; Muley, A.; Kastenhuber, E.R.; Livshits, G.; Tschaharganeh, D.F.; Socci, N.D.; Lowe, S.W. Inducible in vivo genome editing with CRISPR-Cas9. Nat. Biotechnol. 2015, 33, 390–394.
34. Davis, K.M.; Pattanayak, V.; Thompson, D.B.; Zuris, J.A.; Liu, D.R. Small molecule-triggered Cas9 protein with improved genome-editing specificity. Nat. Chem. Biol. 2015, 11, 316–318.
35. Truong, D.-J.J.; Kühner, K.; Kühn, R.; Werfel, S.; Engelhardt, S.; Wurst, W.; Ortiz, O. Development of an intein-mediated split-Cas9 system for gene therapy. Nucleic Acids Res. 2015, 43, 6450–6458.
36. Wright, A.V.; Sternberg, S.H.; Taylor, D.W.; Staahl, B.T.; Bardales, J.A.; Kornfeld, J.E.; Doudna, J.A. Rational design of a split-Cas9 enzyme complex. Proc. Natl. Acad. Sci. USA 2015, 112, 2984–2989.
37. Oakes, B.L.; Nadler, D.C.; Flamholz, A.; Fellmann, C.; Staahl, B.T.; Doudna, J.A.; Savage, D.F. Profiling of engineering hotspots identifies an allosteric CRISPR-Cas9 switch. Nat. Biotechnol. 2016, 34, 646–651.
38. Ran, F.A.; Hsu, P.D.; Lin, C.-Y.; Gootenberg, J.S.; Konermann, S.; Trevino, A.E.; Scott, D.A.; Inoue, A.; Matoba, S.; Zhang, Y.; et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 2013, 154, 1380–1389.
39. Guilinger, J.P.; Thompson, D.B.; Liu, D.R. Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. Nat. Biotechnol. 2014, 32, 577–582.
40. Slaymaker, I.M.; Gao, L.; Zetsche, B.; Scott, D.A.; Yan, W.X.; Zhang, F. Rationally engineered Cas9 nucleases with improved specificity. Science 2016, 351, 84–88.
41. Kleinstiver, B.P.; Pattanayak, V.; Prew, M.S.; Tsai, S.Q.; Nguyen, N.T.; Zheng, Z.; Joung, J.K. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. Nature 2016, 529, 490–495.
42. Fleuren, E.D.G.; Zhang, L.; Wu, J.; Daly, R.J. The kinome "at large" in cancer. Nat. Rev. Cancer 2016, 16, 83–98.
43. Ahmad, G.; Amiji, M. Use of CRISPR/Cas9 gene-editing tools for developing models in drug discovery. Drug Discov. Today 2018, 23, 519–533.
44. Wang, T.; Wei, J.J.; Sabatini, D.M.; Lander, E.S. Genetic screens in human cells using the CRISPR-Cas9 system. Science 2014, 343, 80–84.

45. Shalem, O.; Sanjana, N.E.; Hartenian, E.; Shi, X.; Scott, D.A.; Mikkelson, T.; Heckl, D.; Ebert, B.L.; Root, D.E.; Doench, J.G.; et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science 2014, 343, 84–87.
46. Joung, J.; Konermann, S.; Gootenberg, J.S.; Abudayyeh, O.O.; Platt, R.J.; Brigham, M.D.; Sanjana, N.E.; Zhang, F. Genome-scale CRISPR-Cas9 knockout and transcriptional activation screening. Nat. Protoc. 2017, 12, 828–863.
47. Luo, J. CRISPR/Cas9: From Genome Engineering to Cancer Drug Discovery. Trends Cancer 2016, 2, 313–324.
48. Maeder, M.L.; Linder, S.J.; Cascio, V.M.; Fu, Y.; Ho, Q.H.; Joung, J.K. CRISPR RNA-guided activation of endogenous human genes. Nat. Methods 2013, 10, 977–979.
49. Gilbert, L.A.; Horlbeck, M.A.; Adamson, B.; Villalta, J.E.; Chen, Y.; Whitehead, E.H.; Guimaraes, C.; Panning, B.; Ploegh, H.L.; Bassik, M.C.; et al. Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. Cell 2014, 159, 647–661.
50. Chavez, A.; Tuttle, M.; Pruitt, B.W.; Ewen-Campen, B.; Chari, R.; Ter-Ovanesyan, D.; Haque, S.J.; Cecchi, R.J.; Kowal, E.J.K.; Buchthal, J.; et al. Comparison of Cas9 activators in multiple species. Nat. Methods 2016, 13, 563–567.
51. Konermann, S.; Brigham, M.D.; Trevino, A.E.; Joung, J.; Abudayyeh, O.O.; Barcena, C.; Hsu, P.D.; Habib, N.; Gootenberg, J.S.; Nishimasu, H.; et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature 2015, 517, 583–588.