GENOME ENGINEERING VIA CRISPR-Cas SYSTEMS: INSERTING THE 'PRECISION' IN PRECISION MEDICINE

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Introduction

Genomes encode the instructions for life forms. The ability to change this code in living cells can enable manipulation of organismal function and development. Though this was previously challenging, the recent advent of RNA-guided effectors derived from the clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) systems has dramatically transformed our ability to engineer genomes. Coupled with rapidly advancing synthetic biology toolsets, we now have powerful capabilities to perturb genomes to decipher function, program novel function, and repair aberrant function. With progressive advances, tools based on the CRISPR-Cas systems are creating realistic possibilities to cure various human diseases, and by enabling improved interpretation of the human genome, also shedding powerful insights into new therapeutic targets. These advances ultimately help foster the practice of precision medicine.

The Promise of CRISPR-Cas9: A Clinical Perspective

In prokaryotic organisms, the CRISPR systems function as adaptive mechanisms of defense against pathogens[1]. These systems have been categorized into multiple classes[2], each with different subtypes across multiple organisms and versatile mechanisms of action, of which the type II CRISPR-Cas9 system has been the most widely adapted for RNA-guided double-stranded DNA (dsDNA) targeting with a single effector nuclease. Here, the invading pathogenic viral or plasmid DNA is first cleaved into small fragments, which get incorporated as "spacer" sequences between short repeats within the host CRISPR locus. The locus is transcribed and the resulting transcripts are processed to generate CRISPR RNA (crRNA), which in turn hybridize to a trans-activating crRNA (tracrRNA) and then complex with a large protein Cas9[3]. Guided by the spacer sequence within the crRNA as well as the presence of a protospacer adjacent motif transcribed and the resulting transcripts are

processed to generate CRISPR RNA (crRNA), which in turn hybridize to a trans-activating crRNA (tracrRNA) and then complex with a large protein Cas9[3]. Guided by the spacer sequence within the crRNA as well as the presence of a protospacer adjacent motif (PAM) flanking the crRNA sequence, these complexes target DNA and induce dsDNA cleavage. Recognizing the programmability of these naturally occurring RNA-guided DNA targeting systems, the CRISPR-Cas9 system was subsequently engineered for functioning in eukaryotic cells, including in human cells[4,5], paving the way for powerful and unprecedented genome engineering functionalities. The emergence of this breakthrough genome engineering technology has improved recapitulation, screening, and elucidation of genetic disease mechanisms, thereby bridging the gap between genome sequencing and genome interpretations. This, in turn, affords clinical relevance to applied genomics. The system's remarkable ease of programmability and efficiency have also enabled widespread adaption, surpassing predecessor systems such as metanucleases, zinc finger nucleases (ZFNs) and transcription activator-like effector-based nucleases (TALENs).

Notably, the ability of Cas9 to serve as a programmable recruitment molecule, bringing together RNA, DNA, and protein effectors, enables a range of targeting modalities, including genome editing, regulation, and other novel functions. This has spurred numerous translational research efforts where mutant genes corresponding to monogenic disorders have been successfully corrected in preclinical settings by utilizing homology-directed repair (HDR). Examples include β-Thalassemia[6–9], Cystic Fibrosis[10], Duchenne Muscular Dystrophy[11], and Sickle Cell Anemia[12]. In combination with patient-derived cells, including induced pluripotent stem cells (iPSCs), this may have important implications in cellular replacement therapies. Alternatively, by utilizing the error-prone non-homologous end joining (NHEJ) DNA repair pathway, the system has also been utilized for gene therapy applications. For instance, disruption of CCR5, which is critical for the entry of HIV into cells, can confer host resistance against the virus[13]. Beyond genome editing, the CRISPR-Cas9 system has been harnessed for novel applications, such as gene regulation. Through fusion of a nuclease-null "dead" Cas9 (dCas9) with effector domains, it is possible to induce gene activation[14], repression15, and epigenetic modifications[16], amongst other functions[15]. Unlike the changes resulting from a gene-editing event, dCas9 activity in this way enables controlled gene expression without the introduction of permanent changes to the genome, thus opening novel avenues for therapeutics involving regulation of disease-related genes, as well as for driving cell fate changes. Modification of this system also enables targeting of RNA rather than DNA[17], and has been demonstrated in disease settings such as for Myotonic Dystrophy Types 1 and 2[18], which result from toxic RNA transcripts arising from microsatellite repeat expansions (MRE) often present in noncoding regions of the genome. This approach may therefore provide additional means of controlling similar disease phenotypes when directed in vivo, including for ALS and Huntington's Disease. Nonetheless, an important consideration for prospective dCas9 and RNA-Cas9 therapies is the requirement for continuous administration to the patient, and dosage optimization between patients exhibiting different degrees of disease penetrance. Additionally, despite the relative lack of immunogenicity observed from certain delivery systems, such as adeno-associated viral (AAV) vectors, repeated dosing still poses risk of developing immunogenicity that could vary between patients due to their unique immune repertoire[19]. Regardless of such risks, given the early state of CRISPR-Cas9 based genome editing, such alternative approaches may tremendously reduce risk and alleviate ethical concerns revolving around permanent genome modifications. From the perspective of personalized medicine, this translates into the clinical advantage of providing customizable dosage regulation for each patient.

Additionally, the multiplexing and scalability made possible by the system have also been leveraged to enable large-scale genetic and epigenetic screens to elucidate gene functions and interactions, and characterize non-coding genomic elements[20]. This has enabled systematic and high-throughput unraveling of new targets for therapeutic interventions in various disease settings, such as mapping cancer cell vulnerabilities[21] and dissecting host-pathogen interactions[22].

Taken together, extending the system to target complex genetic disorders, efficaciously engineering in vivo organ systems, and improving overall gene targeting efficiencies will rely on continued basic science research and development efforts. These include, among others, harnessing of novel CRISPR-Cas orthologs, systematic protein engineering, sequence and chemical engineering of guide RNAs, improving homology-directed repair HDR, and importantly, developing new and improved delivery methods. With continued innovations, this system will play an important role in realizing the potential of precision medicine that is customizable to each patient's genetic information. A summary of the rapidly expanding CRISPR-Cas toolbox is presented in the Table below. It is noteworthy that this toolbox highlights some of the expanded capabilities of CRISPR-Cas system and are shown in this table as genome editing and regulation, RNA targeting and diagnostics, sgRNA optimization, cas9 delivery and genetic screens.

Table: The CRISPR-Cas Toolbox

Tools	Functions	Advantages	Limitations
Genome Editing			
Cas9	● Cas9 nuclease associates with a programmable sgRNA molecule for RNA guided targeting of a specific sequence within a genomic locus (or loci, in the case of multiplexed targeting)[1]	● Precision DNA targeting/editing; ● Using orthologs increases PAM sequence versatility and targeting site diversity, and enables combinatorial use. PAM-interacting regions have also been modified for more versatile targeting. ● Screening applications for novel disease-related gene interactions	● Off-target effects that are potentially oncogenic; need to increase specificity ● Higher frequency of NHEJ over HDR
Cas9 nickase (Cas9n)	● Mutation of either the RuvC or HNH domain within Cas9 prevents double-stranded cleavage at the PAM sequence, but enables single-strand "nicking."[24]	● Two Cas9n can be utilized for double-stranded cleavage at a target sequence, leading to improved specificity ● Novel applications requiring just a single-strand nick	● Decreased double-strand break efficiency, due to requirement of 2 Cas9n at each target site
Genome Regulation			
dCas9 and fusions	● Mutation of both catalytic domains, RuvC and HNH, within the Cas9 abolishes endonuclease activity, yielding a "dead" Cas9 (dCas9); however, sgRNA association and DNA targeting capabilities are retained[15] ● Fusions of repressive, activating, methylating, etc. protein domains to dCas9 enable novel epigenetic functions[14–16]	● Non-editing based gene regulation; ● Reversible and enables fine tuning of the system	● Size constraints for in vivo delivery ● Requirement for continuous expression of effectors
RNA Targeting			
RNA Cas9 (RCas9)	● Cas9 associated with sgRNA can target ssRNA, when ssRNA is in trans with additional PAM-presenting oligonucleotides (PAMers)[17]	● Transcriptional level silencing can be achieved ● Enables transcript regulation and trafficking	● Repeated dosing required for continuous knockdown, etc.
RNA-based Diagnostics			
C2c2/ Cas13a	● C2c2 nuclease associates with a programmable sgRNA molecule for targeting of a specific protospacer flanking sequence (PFS) in RNA; upon cleavage of the specific target RNA, C2c2 exhibits promiscuous nuclease activity, cleaving all non-specific nearby RNA sequences[25]	● Applied for highly sensitive RNA-based diagnostics[26]	● Beyond diagnostics, fidelity of C2c2 remains to be evaluated in vivo
sgRNA Optimization			
sgRNA aptamers	● Fusion of RNA apatamers[27] (such as MS2, boxB) ● Truncations of sgRNA[28,29] ● Chemical modifications of sgRNA ● Incorporation of long non-coding RNA elements (ie, Xist) up to 4.8kb in the sgRNA[30–32]	● Ease of programmability with novel function (such as activation, repression, etc.) ● Increased sgRNA stability ● Decreased off-target events ● Enables multiple functions on the same sgRNA	● Additional effector proteins required for desired genome engineering application
Delivery			
AAV delivery of Cas9	● Adeno-Associated Viral based delivery of Cas9	● Gene therapy applications ● Lower immunogenicity ● Versatility of tissue-specific AAVs ● No AAV integration into host genome ● Transduction of both mitotic and post-mitotic cells	● Delivery capacity is limited[33,34] ● Systemic diseases are difficult to target with one AAV serotype
Genetic Screens			
Cas9 or dCas9 based	● Cas9-based genome disruption or dCas9-based genome repression, activation, etc. using a pooled library of sgRNAs enables multiplexed targeting of genes for loss-of-function or gain-of-function screening	● Numerous genes targeted in a single screen ● Applications in discovering novel drug targets, elucidating gene functions and interactions, genomic element functions, and revealing gene fitness[21,22,35] ● CRISPR-Cas systems enable high precision targeting	● Library size limitations ● Screens are conducted in cell lines, which don't fully recapitulate cell biology in vivo

Ongoing or Anticipated Clinical Trials

Though CRISPR-Cas holds promise to revolutionize the treatment of various diseases, current prospects for its direct administration in vivo are still in early stages due to limitations in targeting efficiency, specificity, delivery, and other risk considerations. We anticipate that initial clinical applications will therefore be focused on engineering of in vivo organ systems that are immune privileged (such as the eye, testicular Sertoli cells, placenta and to an extent CNS), and also for ex vivo cellular engineering. Notably, for the latter, the first CRISPR-Cas9 based clinical trials are now underway for a few disease indications, such as enhancing immunotherapy through ex vivo genome engineering of patient derived T-cells23. As ex vivo methods enable monitoring of genetically modified cells prior to reintroduction into patients, they are considered the safest direct therapeutic application of CRISPR-Cas9 systems. Other clinical trials for investigating the safety of CRISPR-Cas9 are also underway.

Finally, though considerable research efforts will be required before realization of CRISPR-Cas systems in precision gene therapeutics, these systems also have an important contribution to pharmacological development. Specifically, the precision of the system coupled with application in genetic screens, as indicated above, is anticipated to further discovery of novel genetic roles, interactions, or biological pathways that are implicated in diseases, and in turn unravel novel therapeutic targets. Taken together, this recent advent of the powerful, versatile, and rapidly advancing CRISPR-Cas toolsets is poised to transform the practice of precision medicine.

References

1. Marraffini, L. A. CRISPR-Cas immunity in prokaryotes. Nature 526, 55–61 (2015).
2.Makarova, K. S. et al. An updated evolutionary classification of CRISPR–Cas systems. Nat. Rev. Microbiol. 13, 722–736 (2015).
3.Deltcheva, E., Chylinski, K., Sharma, C. M. & Gonzales, K. CRISPR RNA maturation by trans -encoded small RNA and host factor RNase III. Nature 471, 602–607 (2011).
4.Mali, P. et al. RNA-Guided Human Genome Engineering via Cas9 Prashant. Science (80-.). 339, 823–826 (2013).
5.Cong, L. et al. Multiplex Genome Engineering Using CRISPR/VCas Systems. Science (80-.). 339, 819–823 (2013).
6.Xie, F. et al. Seamless gene correction of β-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac. Genome Res. 24, 1526–33 (2014).
7.Song, B. et al. Improved Hematopoietic Differentiation Efficiency of Gene-Corrected Beta-Thalassemia Induced Pluripotent Stem Cells by CRISPR/Cas9 System. Stem Cells Dev. 24, 1053–1065 (2015).
8.Xu, P. et al. Both TALENs and CRISPR/Cas9 directly target the HBB IVS2–654 (C > T) mutation in β-thalassemia-derived iPSCs. Sci. Rep. 5, 12065 (2015).
9.Cells, P. S. P LURIPOTENT S TEM C ELLS Na ¨ 1 ve Induced Pluripotent Stem Cells Generated From b -Thalassemia Fibroblasts Allow Efficient Gene Correction With CRISPR / Cas9. 8–19 (2016). doi:10.5966/sctm.2015-0157 In
10.Schwank, G. et al. Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. Cell Stem Cell 13, 653–658 (2013).
11.Li, H. L. et al. Precise correction of the dystrophin gene in duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9. Stem Cell Reports 4, 143–154 (2015).
12.Dewitt, M. A. et al. Selection-free genome editing of the sickle mutation in human adult hematopoietic stem / progenitor cells. 134, (2016).
13.Tebas, P. et al. Gene Editing of CCR5 in Autologous CD4 T Cells of Persons Infected with HIV. N. Engl. J. Med. 370, 901–910 (2014).
14.Mali, P. et al. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nat. Biotechnol. 31, 833–838 (2013).
15.Qi, L. S. et al. Repurposing CRISPR as an RNA-γuided platform for sequence-specific control of gene expression. Cell 152, 1173–1183 (2013).
16.Hilton, I. B. et al. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. Nat. Biotechnol. 33, 510–517 (2015).
17.Nelles, D. A. et al. Programmable RNA Tracking in Live Cells with CRISPR/Cas9. Cell 165, 488–496 (2016).
18.Batra, R. et al. Elimination of Toxic Microsatellite Repeat Expansion RNA by RNA-Targeting Cas9. Cell 170, 899–912.e10 (2017).
19.Chirmule, N. et al. Immune responses to adenovirus and adeno-associated virus in humans. Gene Ther. 6, 1574–83 (1999).
20.Mali, P., Esvelt, K. M. & Church, G. M. Cas9 as a versatile tool for engineering biology. Nat. Methods 10, 957–963 (2013).
21.Shen, J. P. et al. Combinatorial CRISPR–Cas9 screens for de novo mapping of genetic interactions. Nat. Methods 14, 573–576 (2017).
22.Zhou, Y. et al. High-throughput screening of a CRISPR/Cas9 library for functional genomics in human cells. Nature 509, 487–491 (2014).
23.Cyranoski, D. CRISPR gene editing tested in a person. Nature 539, 479 (2016).
24.Ran, F. A. et al. Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity. Cell 154, 1380–1389 (2013).
25.Abudayyeh, O. O. et al. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science (80-.). 353, aaf5573 (2016).
26.Gootenberg, J. S. et al. Nucleic acid detection with CRISPR-Cas13a/C2c2. Science (80-.). 356, 438–442 (2017).
27.Kiani, S. et al. HHS Public Access. 12, 1051–1054 (2015).
28.Dahlman, J. E. et al. HHS Public Access. 33, 1159–1161 (2016).
29.Fu, Y. et al. HHS Public Access. 32, 279–284 (2014).
30.Minks, J., Baldry, S. El, Yang, C., Cotton, A. M. & Brown, C. J. XIST-induced silencing of flanking genes is achieved by additive action of repeat a monomers in human somatic cells. Epigenetics Chromatin 6, 1 (2013).
31.Ørom, U. A. et al. NIH Public Access. 143, 46–58 (2014).
32.Wang, K. C. et al. Long noncoding RNA programs active chromatin domain to coordinate homeotic gene activation. Nature 472, 120–124 (2011).
33.Wu, Z., Yang, H. & Colosi, P. Effect of Genome Size on AAV Vector Packaging. Mol. Ther. 18, 80–86 (2010).
34.Dong, B., Nakai, H. & Xiao, W. Characterization of Genome Integrity for Oversized Recombinant AAV Vector. Mol. Ther. 18, 87–92 (2010).
35.Korkmaz, G. et al. Functional genetic screens for enhancer elements in the human genome using CRISPR-Cas9. Nat. Biotechnol. 34, 192–198 (2016).

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