Expanding the Biologist’s Toolkit with CRISPR-Cas9

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Few discoveries transform a discipline overnight, but biologists today can manipulate cells in ways never possible before, thanks to a peculiar form of prokaryotic adaptive immunity mediated by clustered regularly interspaced short palindromic repeats (CRISPR). From elegant studies that deciphered how these immune systems function in bacteria, researchers quickly uncovered the technological potential of Cas9, an RNA-guided DNA cleaving enzyme, for genome engineering. Here we highlight the recent explosion in visionary applications of CRISPR-Cas9 that promises to usher in a new era of biological understanding and control.

It was only 7 years ago that a fledgling group of international scientists met at the University of California, Berkeley, for the first annual meeting on CRISPRs. A diverse range of expertise was represented—microbiology, biochemistry, metagenomics, food science—allowing the mystery of CRISPR immune system function to be unraveled collectively from multiple lines of experimentation. Each subsequent conference boasted more breakthrough discoveries, and the increasing rate of CRISPR-related publications reflected an intensifying interest in the topic. A description of the molecular function of Cas9 and suggestion of its use for genome engineering, presented at the 2012 meeting, foreshadowed an explosion of research using CRISPR-Cas9 that was soon to come.

Beginning in January 2013, a flurry of studies demonstrated that site-specific DNA editing in eukaryotic cells could be achieved through the heterologous expression of Cas9 together with a guide RNA. Two years and >1,000 publications later (Figure 1A), the technology has gone viral. The genomes of virtually all model plants and animals have been modified with CRISPR-Cas9, and creative new tools continue to expand the capabilities of this system. While CRISPR biology remains an active area of study, the memorable acronym is now more commonly associated with genome engineering than it is with prokaryotic adaptive immunity.

In this perspective, we provide a concise summary of how the CRISPR-Cas9 technology emerged and is enabling remarkable innovations in the biological sciences. We encourage the reader to consult the recent literature for more comprehensive reviews (Doudna and Charpentier, 2014; Hsu et al., 2014; Mali et al., 2013b). Detailed protocols for the numerous applications involving CRISPR-Cas9 can be found in a recent volume of Methods in Enzymology (Doudna and Sontheimer, 2014). Finally, we apologize to our many colleagues whose work we could not discuss due to length constraints, or because the work was published after completion of this article.

“The Biological Significance of These Sequences Is Not Known”

So concluded a study published in 1987, in which the authors inadvertently discovered the first genomic CRISPR locus in Escherichia coli while sequencing the iap gene (Ishino et al., 1987). CRISPRs have since been found in roughly 40% and 90% of all bacterial and archaeal species, respectively (Grissa et al., 2007), and are characterized by short direct repeats interrupted at regular intervals by unique spacer sequences. Yet it wasn’t until 2005 that a potential connection between CRISPRs and antiviral immune defense was established, when multiple laboratories reported that spacers derive from foreign genetic elements (Bolotin et al., 2005; Mojica et al., 2005; Poyrel et al., 2005). A landmark study in 2007 from Barrangou and colleagues provided the definitive link: working in Staphylococcus thermophilus, the authors demonstrated that CRISPR spacers confer potent resistance to bacterial viruses (bacteriophage) bearing matching DNA sequences, and that bacteria could actively vaccinate themselves against bacteriophage by integrating new spacers into the pre-existing CRISPR locus (Barrangou et al., 2007). The central role of noncoding CRISPR RNA (crRNA) in this pathway was revealed shortly thereafter by pioneering work from the van der Oost laboratory (Brouns et al., 2008).

CRISPRs function together with CRISPR-associated (cas) genes that typically flank CRISPR loci in the genome, and the entire pathway is consequently referred to as CRISPR-Cas. Adaptive immunity proceeds in three stages: acquisition (or adaptation), CRISPR RNA biogenesis, and interference (for recent reviews, see Barrangou and Marraffini, 2014; van der Oost et al., 2014). During acquisition, new spacers are selected from foreign nucleic acids and integrated at one end of the CRISPR locus. RNA precursors are then transcribed from the CRISPR locus and enzymatically processed into mature crRNAs, which are bound by one or more Cas proteins to form ribonucleoprotein targeting complexes that each contain a single spacer (guide) sequence. Finally, Cas nucleases cleave target
nucleic acids that are marked for degradation via complementary base pairing to the crRNA.

CRISPR-Cas immune systems have been classified into three types and numerous subtypes based primarily on cas gene phylogeny (Makarova et al., 2011), and while many mechanistic features are widely conserved, significant differences exist. For example, Type I and III systems require only crRNA for targeting, while Type II systems also use trans-activating CRISPR RNA (tracrRNA) (Deltcheva et al., 2011). In addition, the protein composition of crRNA-Cas targeting complexes is highly variable, with complexes in Type I and III systems typically comprising greater than eight subunits (Brouns et al., 2008; Hale et al., 2009). In contrast, Type II systems require only a single polypeptide: Cas9 (Sapranaukas et al., 2011).

RNA-Guided DNA Targeting by Cas9

Cas9 is a DNA endonuclease that generates double-stranded breaks (DSBs) in DNA target sequences identified through base pairing to the guide RNA (Figure 1B) (Gasiunas et al., 2012; Jinek et al., 2012). Cas9 functions naturally with a dual-guide RNA composed of crRNA and tracrRNA (Jinek et al., 2012); the 5’ end of the crRNA base-pairs with target DNA, whereas the 3’ end forms a double-stranded stem with the tracrRNA to facilitate Cas9 recruitment. Because DNA targets are recognized via RNA-DNA base pairing, changing the sequence of the guide RNA easily alters DNA specificity.

Efficient targeting also requires the presence of a short sequence motif proximal to the DNA target sequence, known as the protospacer adjacent motif (PAM) (Mojica et al., 2009). By specifically selecting spacers at PAM sites during acquisition (Yosef et al., 2012), CRISPR-Cas immune systems can discriminate between self and non-self sequences during interference via PAM recognition. Targets found in foreign DNA contain a PAM and are targeted, whereas matching targets in the CRISPR locus itself, from which the crRNA is transcribed, do not contain a PAM and are avoided. Cas9 from Streptococcus pyogenes, which has been the focus of most studies to date, recognizes a 5’-NGG-3’ PAM sequence (Jinek et al., 2012; Mojica et al., 2009).

The ability to edit genomic DNA inside cells has been limited by the dearth of effective tools to introduce site-specific DSBs (Carroll, 2014). Earlier methods relied on protein-only systems such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), but the feasibility of engineering these designer enzymes to recognize new sequences was limited. In contrast, the CRISPR-Cas system has the distinct advantage of relying on RNA for specificity. And while Cas9 shares molecular capabilities with other CRISPR-Cas systems, its compositional simplicity has been paramount to its successful application. Not only does it encompass only a single polypeptide, but remarkably, it retains full activity with a chimeric single-guide RNA (sgRNA), generated by connecting the 3’ end of the crRNA to the 5’ end of the tracrRNA (Jinek et al., 2012).

Genome Editing with CRISPR-Cas9

Six months after the first description of the molecular function of Cas9 (Jinek et al., 2012), six studies demonstrated that Cas9 together with sgRNA can be used to specifically edit the genomes of human cells (Cho et al., 2013; Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013c), zebrafish embryos (Hwang et al., 2013), and bacteria (Jiang et al., 2013). The technology was rapidly extended to other model organisms, and by May of 2013, the Jaenisch laboratory reported the one-step generation of mice mutated at multiple alleles via zygotic injection of Cas9 mRNA and sgRNAs (Wang et al., 2013). What had once been laborious and time-consuming was now facile and rapidly achievable.
In the simplest embodiment of the CRISPR-Cas9 technology (Figure 1C), the sgRNA guide sequence is designed to target a complementary 20-bp site flanked by NGG within the gene of interest. Heterologous expression of Cas9 together with sgRNA may be accomplished by stable lentiviral transfection, transient plasmid transfection, direct DNA or RNA injection, or transfection with purified ribonucleoprotein complex, with the optimal strategy depending on the desired application and the cell or organism being edited. After trafficking to the nucleus through an appended nuclear localization signal, Cas9 targets the locus of interest and induces a DSB that is repaired by the cell’s endogenous machinery via non-homologous end-joining (NHEJ). Because NHEJ is an error-prone repair pathway that results in small insertions or deletions (indels), the open reading frame is disrupted and the gene becomes inactivated. While the editing efficiency with CRISPR-Cas9 can be as high 80% (Kim et al., 2014; Zuris et al., 2015), it is cell and site specific and depends on the delivery method. In addition, the resulting cell population will be inherently heterogeneous, both in the percentage of cells that were edited and in the specific genotype of the edited cells.

The genome can also be edited in a more precise manner using homology-directed repair (HDR). By combining Cas9-sgRNA delivery with a donor DNA that bears homology to sequences flanking the targeted site, DSBs are repaired using the donor DNA as a template. Importantly, this strategy enables new sequences to be introduced into the gene of interest, such as epitope tags, and specifically defined mutations to be installed, such as those that might mimic or correct disease-causing alleles. However, efficiencies of HDR are significantly lower than NHEJ, and more work is needed to develop strategies that bias the cell’s natural DNA repair machinery toward the desired outcome.

Genome editing with CRISPR-Cas9 is now a routine procedure for virtually all model plants and animals, and recent progress has pushed the technology into even more interesting directions. A unique advantage of CRISPR-Cas9 over earlier genome editing methods is that multiplexable targeting is easily achieved by co-expressing Cas9 with multiple sgRNAs simultaneously. In addition to editing multiple chromosomal loci in a single experiment with this approach, large chromosomal deletions can be achieved by using two sgRNAs to induce DSBs at sites that flank the region of interest (Xiao et al., 2013). Furthermore, large-scale chromosomal rearrangements resembling those found in specific tumors can be introduced (Choi and Meyerson, 2014; Torres et al., 2014). Indeed, CRISPR-Cas9 offers great promise in transforming the tools available to recreate, model, and treat human cancers (Maddalo et al., 2014; Platt et al., 2014; Sánchez-Rivera et al., 2014).

**Leveraging CRISPR-Cas9 as a Versatile DNA-Binding System**

Cas9 generates DSBs using two conserved nuclease domains (HNH and RuvC) that cleave both strands of DNA target sequences. Inactivating both catalytic active sites via point mutations results in catalytically inactive Cas9 (dCas9), which remains fully active for programmable, RNA-guided DNA binding (Jinek et al., 2012). Numerous studies have taken advantage of this discovery to develop powerful new tools for regulating gene expression.

When expressed in bacteria, dCas9 together with sgRNA can sterically occlude RNA polymerase from binding promoter sequences and thereby downregulate the expression of specific transcripts (Bikard et al., 2013; Qi et al., 2013). More robust gene expression control in eukaryotes becomes possible by fusing Cas9 to specific effector domains, such as transcriptional repressors and activators, which are recruited to specific genomic loci via the sgRNA (Gilbert et al., 2013; Maeder et al., 2013; Perez-Pinera et al., 2013). Recent developments continue to increase the efficiency and dynamic range of gene regulation by CRISPR-Cas9. In addition to improvements in the sgRNA design (Chen and Huang, 2014), newer methods involve recruiting multiple effector proteins through engineered molecular scaffolds fused to Cas9 (Gilbert et al., 2014; Tanenbaum et al., 2014) or RNA aptamers fused to sgRNA (Konermann et al., 2015; Mali et al., 2013a).

dCas9 can also be used to probe and manipulate the genome in other ways that ultimately rely on specific nucleic acid targeting. For example, DNA loci can be imaged in live cells using dCas9-GFP fusions (Chen et al., 2013), offering new insights into the dynamics and conformation of genomic loci. Robust genetic circuits can be constructed and implemented using dCas9-mediated transcriptional control (Kiani et al., 2014; Liu et al., 2014; Nissim et al., 2014), providing valuable new regulatory devices for synthetic biology applications. dCas9 fusions to effector domains that install epigenetic markers may also enable specific perturbation of epigenetic regulation. Finally, a recent report demonstrated that dCas9 can bind single-stranded RNA targets (O’Connell et al., 2014), suggesting that programmable manipulation of cellular RNA transcripts may become possible in the near future.

**High-Throughput Screening Using CRISPR-Cas9**

CRISPR-Cas9 enables facile, targeted perturbation of specific genes in the cell, either through permanent genome editing or temporary gene regulation. A systematic investigation of gene function, however, requires this level of control to be extended across the genome. A number of recent studies have harnessed the programmable nature of CRISPR-Cas9 to conduct powerful genome-scale screens. Importantly, this approach enables the hypothesis-free discovery of novel pathways that underlie a given biological process.

Using lentiviral sgRNA libraries and catalytically active Cas9, loss-of-function gene knockout screens were performed in both human and mouse cells (Koike-Yusa et al., 2014; Shalem et al., 2014; Wang et al., 2014a; Zhou et al., 2014). Deep sequencing of the sgRNA pool after either positive or negative selection revealed genes essential for cell viability, as well as genes involved in resistance to specific small-molecule drugs. While focused libraries will prove useful for targeted screens in which the candidate genes are selected by the researcher, genome-wide libraries that query all protein-coding genes will have a greater likelihood of discovering novel hits that were not previously identified (Koike-Yusa et al., 2014; Shalem et al., 2014).

Catalytically inactive dCas9 has also been co-opted for genome-scale screening by directly upregulating or downregulating gene expression (Gilbert et al., 2014; Konermann et al., 2015). In comparison to the indels generated by active Cas9,
which may be insufficient to inactivate non-coding RNAs or disrupt a given open reading frame, transcriptional silencing by dCas9 can more effectively block gene expression in some contexts. However, the ability to perform gain-of-function screens using dCas9-mediated recruitment of transcriptional activators is arguably the most significant advantage of this approach over active Cas9 and has not been possible with earlier technologies.

**Off-Target Effects of CRISPR-Cas9**

The success of any genome engineering technique, either as a basic research tool or in therapeutic applications, will ultimately be limited by its specificity. Early reports warned that CRISPR-Cas9 causes frequent off-target editing events (Fu et al., 2013), leading many laboratories to analyze cleavage specificity more thoroughly using different approaches (Hsu et al., 2013; Mali et al., 2013a; Pattanayak et al., 2013). While positions throughout the 20-bp target sequence affect specificity, mismatches encountered proximal to the PAM, within a seed sequence of ~8–12 nucleotides, have the largest impact on cleavage accuracy. These findings are consistent with the mechanism of DNA interrogation by Cas9, in which the duplex is unwound beginning at the PAM, in a directional manner that depends on RNA-DNA complementarity (Sternberg et al., 2014; Szczelkun et al., 2014).

A number of strategies have been developed that reduce off-target effects. Requiring two independent Cas9 binding events for genome editing effectively increases the length of DNA being recognized and has been accomplished in two ways. First, a nickase variant of Cas9 can be used, in which only one active site is mutated (Mali et al., 2013a; Ran et al., 2013). Pairs of sgRNAs then direct Cas9 to nick two closely spaced target sites to mimic a DSB; off-target nicking events with just a single sgRNA are precisely repaired without indel formation. Second, dCas9-FokI fusions can be used, similarly to ZFNs and TALENs (Guilinger et al., 2014; Tsai et al., 2014). Pairs of sgRNAs direct dCas9-FokI to two adjacent target sites, and a DSB is generated upon FokI dimerization; off-target binding events with a Cas9-FokI monomer do not result in cleavage. Finally, truncated sgRNAs, which contain shorter regions of complementarity to the target, have been shown to reduce off-target cleavage events without sacrificing on-target editing efficiencies (Fu et al., 2014). While most specificity studies to date have restricted their analysis to predicted off-target sites, two recent reports applied unbiased, whole-genome sequencing and found that the incidence of off-target mutations may be sufficiently low for certain applications (Smith et al., 2014; Veres et al., 2014).

Recent ChIP-seq studies have revealed that DNA binding by Cas9 is far more promiscuous than DNA cleavage (Cencic et al., 2014; Duan et al., 2014; Kucscu et al., 2014; Wu et al., 2014). The relevance of these findings for gene regulation applications involving dCas9 remains unclear, since off-target binding events may be too transient to affect transcription. Notably, a careful analysis of dCas9-mediated transcriptional repression found minimal off-target activity from properly designed sgRNAs (Gilbert et al., 2014). Nevertheless, in vitro experiments confirm that off-target DNAs with mismatches distal from the cleavage site can be tightly bound but not cleaved (Sternberg et al., 2014). The molecular cues that regulate catalytic activity have yet to be fully determined.

**Future Directions of CRISPR-Cas9 Technologies**

Cas9 holds great promise as a therapeutic strategy to treat human genetic diseases, as evidenced by the recent emergence of numerous companies dedicated to this cause. In proof-of-concept experiments, a disease-causing Fah mutation was successfully corrected in adult mice by hydrodynamic injection of a donor DNA template and plasmid DNA encoding Cas9 and sgRNA (Yin et al., 2014), and Duchenne muscular dystrophy was prevented by direct injection of Cas9 mRNA, sgRNA, and donor DNA template into the mouse germline (Long et al., 2014). Significant hurdles exist before similar experiments can be performed on human patients, but successes in ZFN-based human clinical trials demonstrate the exciting potential of general approaches using programmable nucleases (Tebas et al., 2014).

A number of recent studies have highlighted the ability of CRISPR-Cas9 to specifically alter ecological populations. Within microbial communities, Cas9 was packaged in bacteriophage and programmed to selectively kill virulent bacteria by targeting virulence genes, while leaving other bacteria unaffected (Bikard et al., 2014; Citorik et al., 2014). In animal populations that undergo sexual reproduction, Cas9-based gene drives could be used to rapidly spread altered traits and control invasive species (Esvelt et al., 2014). Finally, CRISPR-Cas9 can be used to genetically improve major staple crops such as bread wheat (Wang et al., 2014b). Many of these applications will require renewed attention to existing and future regulatory challenges (Oye et al., 2014; Voytas and Gao, 2014).

Finally, there is still significant room for basic tool development in the CRISPR-Cas9 technology space. Recent high-resolution structures of Cas9 in both unbound and DNA-bound states have been particularly insightful for the rational design of new Cas9-sgRNA variants and will surely inform future proteint engineering efforts (Anders et al., 2014; Jinek et al., 2014; Nishimasu et al., 2014). Advances in our biochemical understanding of DNA recognition by CRISPR-Cas9 have inspired strategies to target new nucleic acids substrates such as single-stranded RNA (O’Connell et al., 2014). And an exciting avenue of future research will be the characterization and application of naturally occurring Cas9 homologs for genome engineering beyond those already described (Esvelt et al., 2013; Hou et al., 2013). For example, smaller variants may be more easily delivered with viral vectors, and orthogonal sgRNA and PAM specificities will enable a wider range of multiplexable outputs, including the simultaneous upregulation and downregulation of gene expression.

**Conclusions**

The remarkable speed at which the CRISPR-Cas9 technology has spread throughout the biological community attests to its substantial impact in transforming our ability to manipulate cells (Figure 1C). Genome engineering with Cas9 and sgRNA has become so routine that soon, the CRISPR-Cas9 method for editing chromosomal sites in model organisms will require no more attention in research articles than that accorded to PCR and
molecular cloning. Indeed, the ease with which this technology can be practiced, and its tremendous utility, suggests that CRISPR-Cas9 will increasingly become a tool of choice for the next generation of biologists.

REFERENCES


