Advances in Genome Engineering: The CRISPR/Cas9 Revolution

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The ability to specifically alter and regulate gene in living organisms has long been a tantalising prospect in the field of cellular biology ever since the initial descriptions of how DNA encoded genetic information. The 2012 discovery of the RNA-guided DNA endonuclease activity of the bacterial Cas9 protein has spurred the beginning of a genome editing revolution which has attracted considerable attention outside the field of genetics alone. The CRISPR/Cas9 technology has usurped previous gene editing techniques and allowed researchers to explore new areas of genome engineering. The earliest discoveries concerning the clustered, regularly interspaced, short palindromic repeat (CRISPR) loci in prokaryotes led to the subsequent birth of the CRISPR/Cas9 genome editing tool and with it the most profound gene editing technology discovered to date. Novel attempts to improve the precision and efficiency of CRISPR/Cas9 are detailed and recent breakthroughs in the discovery of other CRISPR proteins are highlighted. The ease-of-use and efficiency of CRISPR/Cas9 will more than likely continue to expand our knowledge of genome function and potentially lead to its application in gene therapy for human disease.

Introduction

Genomic DNA sequences encode chemical databases for directing a vast array of biochemical processes. The human genome alone contains a staggering $3x10^9$ base pairs. Elucidating how this genome is regulated and expressed is of utmost importance for comprehending, predicting, and preventing disease. Central to our ability to investigate genome function is the power to test hypotheses by disrupting sequences implicated in a given process. The ability to alter or excise sequences complicit in disease states offers a powerful tool for potentially curing devastating genetic disorders. Controlled and targeted manipulation of DNA in this manner is being ushered in by widespread use of a new gene editing technology, CRISPR/Cas9, which is poised to transform our understanding of the complex interplay between genetics and disease states.

Targeted genome editing necessitates the creation of a double-stranded break (DSB) at a specified DNA sequence. Two different repair pathways predominate in cellular repair of DSBs. Non homologous end-joining (NHEJ) causes insertion/deletion mutations (indels) which can perturb reading frames and DNA regulatory elements, whilst homology-directed repair (HDR) employs the use of "donor" DNA molecules for repairing genetic sequences (Sancar *et al.*, 2004). Prior to CRISPR, previous methods for inducing DSBs at target loci relied on either zinc-finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs), both of which comprise a customisable DNA binding protein domain fused to a nuclease domain.

Despite their potential, widespread adoption of ZFNs and TALENs was prevented by the expense and complexity of protein engineering (Sander and Joung, 2014). The 2012 discovery that *Streptococcus pyogenes*-derived CRISPR-associated protein 9 (Cas9) could induce targeted DSBs based on simple complementary base pairing between an RNA molecule and a target DNA sequence has led to a sustained uptake by research groups in the field of genome editing and engineering. Combining the relative ease of array-based oligonucleotide synthesis with the RNA-guided DNA endonuclease activity of Cas9 has allowed CRISPR technology to dwarf previous genome editing technologies. Twenty years of research assessing a specific prokaryotic loci has resulted in a genome engineering revolution which has seen the applications of CRISPR technology in research increase significantly in a relatively short period of time (Figure 1).

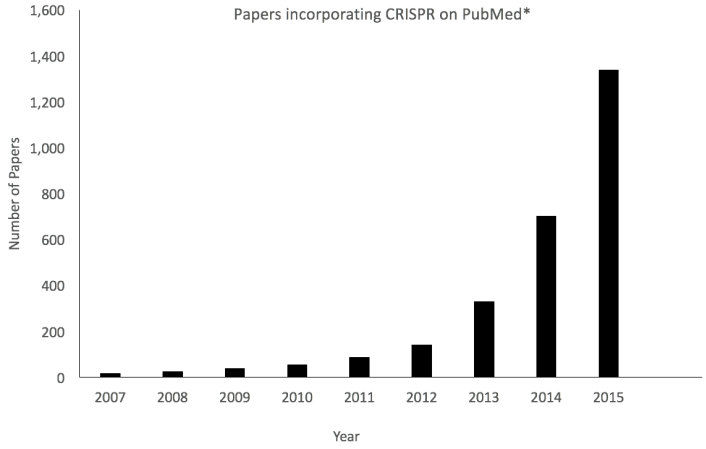

Figure 1. *Significant increase in the study and application of CRISPR technology. CRISPR research has seen an exponential growth since it was first described for genome editing in 2012. A PubMed search was conducted for the term "CRISPR", all primary papers and reviews returned were filtered by year and included.*

From peculiar prokaryotic sequence to robust genome-engineering tool: The CRIPSR story

Sequencing the *iap* gene from *Escherichia coli*, Ishino *et al.* (1987) noticed an unusual genetic element consisting of a repeated pattern of 29 conserved base pairs with a weakly palindromic nature separated by 32 nucleotides of a variable sequence. Genome sequencing advances in the 1990s led to the discovery of similar such sequences in a range of bacterial and archaeal organisms including *Mycobacterium tuberculosis* (Kamerbeek *et al.*, 1997), *Haloferax mediterranei* (Mojica *et al.*, 1995), and *Methanococcus jannaschii* (Bult *et al.*, 1996). Mojica *et al.* (2000) proposed grouping them together into a family of similar sequences defined by the presence of short, often palindromic nucleotide stretches separated by highly diverse "spacer" sequences. The CRISPR acronym (clustered, regularly interspaced, short palindromic repeats) was born two years later, in tandem with the designation *cas* (CRISPR-associated genes) to delineate the operon of genes found in close proximity to CRISPR arrays (Jansen *et al.*, 2002). Bolotin *et al.* (2005) revealed that spacer sequences of *Streptococcus thermophilus* CRISPR arrays are homologous to those found in *S. thermophilus*-specific phages and plasmids whilst Barrangou *et al.* (2007) showed that *S. thermophilus* is capable of directly incorporating short sequences of a phage genome (the protospacer) into the spacer region of its CRISPR array. Such acquisition confers protection against phages containing sequences

identical to the acquired one, revealing that CRISPR is the prokaryotic analogue of an adaptive immune system capable of targeting extra-chromosomal genetic elements (Figure 2).

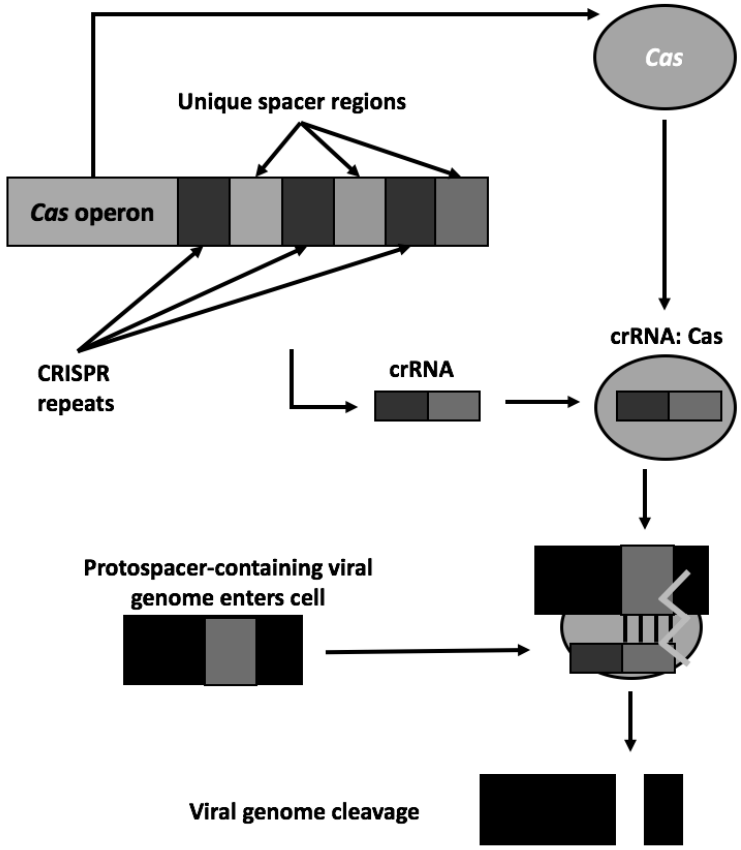

Figure 2. *CRISPR is a prokaryotic adaptive immune system which can be adapted for targeted and precise induction of double-stranded DNA breaks. In prokaryotes, CRISPR/Cas functions as an anti-viral defence mechanism whereby DNA-encoded portions of previous viral encounters (spacers) are used to target and cut viral genomes at precise sequences (protospacer) upon re-exposure.*

Brouns *et al.* (2008) showed that in *E. coli* the CRISPR array was first transcribed into a large pre-crRNA, which was then cleaved into smaller crRNAs containing both a spacer and repeat regions. In addition, Mojica *et al.,* (2009) revealed that small tri-

or di-nucleotide species-specific protospacer-adjacent motifs (PAMs) needed to be located upstream of the target sequence for effective phage defence, while Garneau *et al.* (2010) demonstrated that CRISPR/Cas targets foreign DNA by complementary base-pairing, resulting in the creation of DSBs with blunt ends. Differential RNA sequencing in *S. pyogenes* exposed the existence of small *trans*-encoded RNAs (tracrRNAs) in Type II CRISPR systems which hybridize to the repeat region of pre-crRNA at a 25 nucleotide sequence to form an RNA duplex held together by the Cas9 protein (Deltcheva *et al.*, 2011). Subsequent cleavage of the pre-crRNA produces an active crRNA:tracrRNA complex whereby the 20 nucleotide spacer sequence in the 5′ end of the crRNA portion can target complementary DNA sequences for Cas9-mediated cleavage (Figure 3). This two RNA/one cas protein mechanism contrasts with that of Type I and Type III CRISPR systems where a large multi-protein complex is required to process pre-crRNA (Charpentier *et al.*, 2015). Jinek *et al.* (2012) promptly capitalised upon this unique feature of Type II systems by capturing the *S. pyogenes* crRNA:tracrRNA interaction in a single chimeric "guide RNA" molecule (gRNA). Changes to the 20 nucleotide spacer region of the gRNA were sufficient to target the Cas9 endonuclease to multiple sites within a bacterial plasmid, provided they were downstream of the *S. pyogenes* PAM. This pivotal research provided proof-of-principle that a programmable Cas9-guided gRNA could theoretically be targeted to any PAM-adjacent portion of the genome and served as the impetus for the current CRISRP/Cas9 genome engineering revolution (Figure 4).

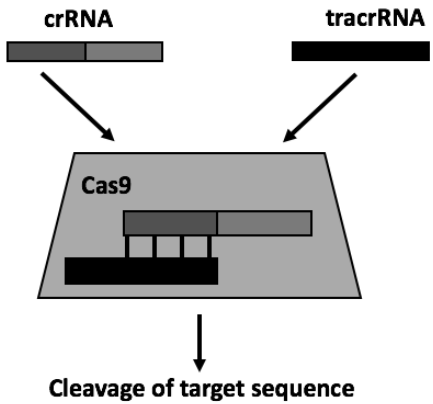

Figure 3. *Type II CRISPR systems harness Cas9 endonucleases to cleave dsDNA breaks. In Type II CRISPR systems, a crRNA:tracrRNA complex guides the Cas9 endonuclease to cleave target sequences. Adapted from Jinek et. al (2012).*

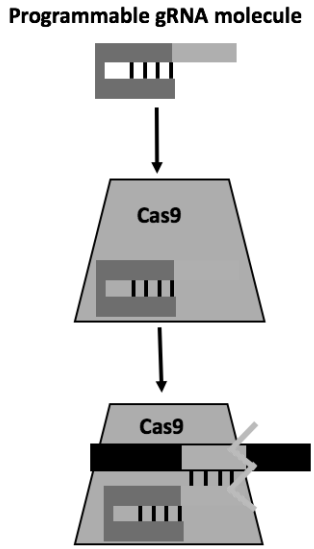

Figure 4. *The Type II CRISPR mechanism uses a guide RNA to direct the Cas9 endonuclease. The basic components of Type II CRISPR systems can be captured in a single guide RNA molecule (gRNA), whereby changes to the 20 nucleotide protospacer region can direct the Cas9 endonuclease to any PAM-adjacent portion of a target genome. Adapted from Jinek et. al (2012).*

Expanding the potential of CRISPR

Studies published in early 2013 demonstrated the versatility of CRISPR/Cas9 in a wide array of cell types. Jiang *et al.* (2013) showed that a Type II CRISPR system could be used to edit the endogenous genes of *E. coli* while Mali *et al.* (2013) engineered a human codon-optimized version of Cas9 encompassing a nuclear localisation signal (NLS) for eukaryotic gene editing. The capabilities of the CRISPR/Cas9 technology continued to be explored as numerous research teams employed it to edit zygotic genomes and create precise gene-edited analogues of key model organisms including *Arabidopsis thaliana* (Li *et al.*, 2013), *Caenorhabditis elegans* (Friedland *et al.*, 2013) , *Mus musculus* (Shen *et al.*, 2013), and *Macaca fascicularis* (Niu *et al.*, 2014). Introduction of stable and heritable changes in embryonic genomes using CRISPR/Cas9 has expedited the time and cost of creating transgenic animals whose study has traditionally been central to exploring the intertwined relationship between genetics and processes such as development and disease progression.

Several of the earliest studies demonstrating the genome-editing power of CRISPR/Cas9 anticipated its eventual use to correct causative mutations in heritable genetic disorders. Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder resulting in the absence of a functional dystrophin, a muscle protein, and causes decreased muscle cell integrity and usually death by the age of thirty from respiratory and cardiac failure (van Deutekom and van Ommen, 2003). Due to the considerable size of the dystrophin gene, it is refractory to traditional viral-mediated gene therapy. Long *et al.* (2014) employed a CRISPR-mediated gene editing therapy to correct the causative genetic lesion in embryos of the DMD mouse model. Subsequent to this, Ousterout *et al.* (2015) used a multiplexing approach comprising several gRNAs to delete ten deleterious exons in a "mutation hotspot" region of the dystrophin gene in cultured myoblast cells derived from human DMD patients. Exon deletion maintained the reading frame of the gene and resulted in the expression of a truncated yet functional dystrophin protein capable of maintaining correct localisation *in vivo* when engrafted onto immunodeficient mice. Furthermore, three research teams independently reported that adeno-virus associated (AAV) vectors could deliver the components of a CRISPR/Cas system for correction of a mutated dystrophin gene in adult mice (Nelson *et al*., 2015, Tabebordbar *et al*., 2015, Long *et al*., 2015). The novelty of these three studies was their systemic delivery of CRISPR/Cas9 gene editing components to treat fully grown animals with a genetic disease, potentially paving the way for its eventual use in DMD-afflicted patients.

Beyond gene editing: Repurposing CRISPR

The genome targeting function of Cas9 has been repurposed beyond its endogenous DNA endonuclease role to equip researchers with new tools to alter gene transcription and map genetic networks. Point mutations in the endonuclease domains of Cas9 create a catalytically inactive version (dCas9) which can remain bound to target sequences and serves as a convenient RNA-guided DNA binding platform. A technique christened CRISPR interference (CRISPRi) can target dCas9 to promoter and coding regions in *E. coli* to downregulate gene expression by physically hindering transcriptional factor recruitment (Qi *et al*., 2013). Eukaryotic transcriptional regulation however is more complex and involves distal and proximal regulatory elements including silencers, enhancers, and histone modifications. Gilbert *et al.* (2013) engineered a version of dCas9 fused to a transcriptional repressive domain to knock down protein expression in human cell lines, obtaining repression levels similar to that obtained using RNA interference (RNAi). Doudna and Charpentier (2014) have suggested that CRISPRi may be particularly potent in eukaryotic cells because it does not compete with endogenous gene knockdown pathways as in RNAi. Fusion of the catalytic core of a p300 acetyltransferase to dCas9 can also induce targeted histone acetylation at enhancer elements to activate nearby gene expression (Hilton *et al*., 2015).

The determination of the crystal structure of Cas9/gRNA with target sequences has also facilitated a more rational and structural approach to improving dCas9 by optimizing the location of associated effector domains (Nishimasu *et al.*, 2014). Konermann *et al.* (2015) built upon this crystal structure by fusing three activation domains to dCas9 to develop a potent transcription activation system termed synergistic activation mediator (SAM), creating a genome-wide gRNA library to identify novel gain-of-function mutations involved in resistance to a melanoma inhibitor. In a manner analogous to genome-wide association studies (GWAS), genome-wide CRISPR genetic perturbation studies will enable researchers to perform unbiased screenings to identify key components of cellular processes.

Improving reliability and efficiency: Next generation CRISPR techniques

The wide range of uses for CRISPR technology is an expanding area of research (Figure 5), but requires improvement in efficacy and reliability. One of the most important issues relating to the application of CRISPR is to what extent the gRNA can tolerate mismatches with the complementary sequence and thus to what extent Cas9 is able to induce unintended off-target DNA cleavage events. Hsu *et al.* (2013) demonstrated that PAM-proximal mismatches were better tolerated than mismatches distal to the PAM, in agreement with work done by Sternberg *et al.* (2014) showing that both gRNA-DNA duplex formation and DNA strand separation are initiated at the PAM. Hsu *et al.* (2013) proposed that a "seed sequence" of 8-14 nucleotides proximal to the PAM was critical for precise DNA targeting and that whether mismatches were adjacent to one another or interspaced affected the promiscuity of the gRNA. Furthermore, Fu *et al.* (2013) showed that off-target sites with up to five mismatches could be cleaved in a human cell-based reporter assay and most noticeably that there were often higher editing levels at predicted off-target sites than the intended on-target sites. Refuting this, Smith *et al.* (2014) found a low frequency of off-target effects in human stem cells using an unbiased and non-predictive whole genome sequencing approach, although it is possible that cell type architecture may affect the on/off-target ratios. Since there could potentially be thousands of off-target sites in the human genome if up to five mismatches were tolerated in a 20 base pair sequence, a clearer picture for how to both account for and avoid off-target effects will need to emerge before any CRISPR therapies can enter human clinical trials.

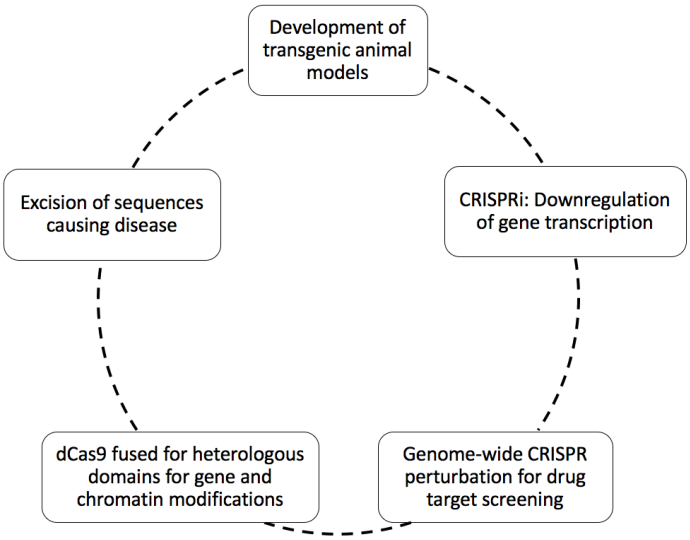

Figure 5. *Multitude of Uses for CRISPR/Cas9 Technology. CRISPR has been used for various applications in a wide range of cell types, animal models, scientific applications and has significant potential to revolutionise each of the areas detailed.*

Ran *et al.* (2013) adopted a "paired nickase" approach for reducing off-target mutagenesis whereby only one of the endonuclease domains of Cas9 is mutated (Cas9n) to produce a nickase capable of cutting only one strand of the DNA. Pairing nickases with offset guide RNAs effectively doubles the number of target bases and thus reduces the number of potential off-target. When a Cas9n/gRNA does nick an off-target site, these single nicks can be repaired by highly efficient base excision repair (BER) with concomitant low levels of unwarranted indel mutations (Mali *et al.*, 2013a). Adaptation of other CRISPR endonucleases for genome engineering purposes may also help to improve editing efficiencies. Ran *et al.* (2015) isolated a Cas9 orthologue from *S. aureus* (SaCas9) which is smaller than the more widely used S. pyogenes Cas9 and thus may be more compliant for cloning into size-restricted viral vectors. Zetsche *et al.* (2015) also isolated a novel CRISPR endonuclease called Cpf1 (CRISPR from *Prevotella* and *Francisella* 1) which does not require a tracrRNA and induces a DSB with a 4 or 5 base pair 5′ overhang. Use of Cpf1 may simplify the design of guide RNAs due to the lack of tracrRNA requirement.

Conclusions

The initial discovery of CRISPR underscores the prime importance of research into basic cellular mechanisms and how we might utilise such research tools for eventual biomedical advances. However for all the excitement that CRISPR/Cas9 has generated, it has garnered a host of concomitant ethical questions concerning its application. The announcement in April 2015 that Chinese researchers had used CRISPR/Cas9 to edit human embryos with considerable off-target effects compelled the National Institutes of Health (NIH) to declare that they would not fund any CRISPR applications involving human embryos (Liang *et al*., 2015). Prof. Doudna, head of one of the research teams which initially described the adaptation of CRISPR systems for genome editing, has referred to the amount of CRISPR/Cas9 papers as a "tsunami" and called for increased education and reflection among scientists as to the sociological and ethical implications of quick and efficient genome editing (Doudna, 2015).

As the use of CRISPR continues to push gene editing boundaries, a measured approach will be required to balance the numerous advantages of such a powerful genetic tool against growing ethical concerns over the fast-paced application of the technology, particularly in the absence of universally agreed upon limits to gene editing. Despite this, it is becoming increasingly apparent that continued improvement of CRISPR systems will enable researchers to interrogate genetic networks on a scale previously unattainable with other technologies and to potentially further our knowledge and treatment of a wide range of complex human diseases.

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