CHAPTER 8

Second Generation Genome Editing Technologies in Drug Discovery

Thomas Malcolm, David A. Mitchell, Niraj Muni
Excision BioTherapeutics Inc., Tarrytown, NY, United States

INTRODUCTION

The most common genetic approach of directly linking a disease state with a genomic mutation is to modify the DNA sequence of a cell (and hence an organism) and observe the effects of this change on the appearance, life cycle or biochemical make-up of the organism. This reverse genetic tactic's significance for modern biology is its simplicity, unlike that of forwarding genetics where the characteristics of a disease state are first observed, and then its genetic abnormality studied. Among the critical requirements of reverse genetic analysis is the ability to modify the DNA genomic sequence of the organism. Genome editing is a type of genetic engineering in which DNA is inserted, deleted, modified or replaced in the genome of living cells and whole organisms.

As of 2018, the accepted methodology for genomic editing of DNA utilises 'molecular scissors' in the form of nucleic acid nucleases. These nucleases can generate specific double-strand breaks (DSBs) at the desired sequence location(s) contained within the genome (Adli, 2018; Doudna and Charpentier, 2014; Haurwitz et al., 2010; Hsu et al., 2014; Wright et al., 2016, 2017; Wu et al., 2015; Yang et al., 2011). The cellular machinery can refurbish the induced DSBs using either nonhomologous end joining (NHEJ) or homologous directed recombination (HDR) (Wu et al., 2015; Farboud and Meyer, 2015; Jasin et al., 1985; Moore and Haber, 1996; Ran et al., 2013; Resnick, 1976; Roth and Wilson, 1986; Szostak et al., 1983). However, as these cellular mechanisms for DNA repair are not perfect, mutations have a relatively high frequency of occurrence due to the low efficiency and precision of a procedure that is a drain on lab personnel and resources.

Whereas in microbiology appropriate genetic engineering tools have been available for decades, the newly discovered genome editing tool, CRISPR-Cas, is revolutionising the way the scientific community designs experiments and focusses attention on disease states in whole animal model systems (Doudna and Charpentier, 2014; Hu, 2017). CRISPR-Cas has been efficiently used in yeast, flies, nematodes and mammals to generate model organisms (Farboud and Meyer, 2015; Akhmetov et al., 2018; Bao et al., 2018; Bassett et al., 2013; Friedland et al., 2013; Gratz et al., 2013a; Lancrey et al., 2018; Li et al., 2013a). The methodology is a more simplified genome-editing approach and has been

Second Generation Cell and Gene-based Therapies
ISBN 978-0-12-812034-7
https://doi.org/10.1016/B978-0-12-812034-7.00008-X

embraced as the premier targeted genome-editing technology (Pattanayak et al., 2014; Ul Ain et al., 2015). In this chapter, we survey the flexibility of the CRISPR-Cas system, namely the Editor function, in model organism construction and drug discovery.

Gene Editing and Drug Discovery

A contributor to druggability is the number of known upstream effectors of the target. The drug discovery industry has focussed on upstream targets using approaches such as activation/inactivation with small molecules and biologics (antibodies and enzymes), interfering RNA (miRNA, shRNA, RNAi), stem cell therapy (embryonic, mesenchymal, haematopoietic, etc.), and classical gene-editing nucleases (zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs)). The field has gradually grown from systemic upstream approaches, progressing from small molecules derived from large library high throughput screens to antibodies, to RNAi approaches, to stem cell treatments. The trend has been to develop tools that move toward the genetic source.

Small Molecules

In general, most small molecule therapeutics target proteins that are related to the disease state, which elicits a cellular/tissue response or cellular/tissue apoptosis to benefit the patient. The roots of the pharmaceutical industry are sewn by small molecule therapeutics, and they continue to play an influential role as industry drivers. Over the past century, much of the industry has advanced its infrastructure to accommodate the chemical engineering and discovery development processes of small molecules. Although many of the small molecule discovery platforms are 'hyper advanced' (comparing today's standards to yester-year platforms), the cost of discovery before preclinical cellular testing remains considerably high in the tens of millions of dollars range (Campbell et al., 2018; Cichonska et al., 2015), with the cost of developing a novel pharmaceutical having been estimated to reaching approximately $1.4 to $2.6 billion in 2017 (DiMasi et al., 2016). The high cost of small molecule discovery can be attributed to complex discovery and screening platforms involving continual innovations such as advanced robotics systems, coupled with more stringent regulatory parameters.

Biologics (Antibodies)

Antibodies are utilised in two ways. First, antibodies are used to target biomarkers or to deliver a payload. For example, antibodies can be attached to a nanoparticle or conjugated directly to a drug (Khoshnejad et al., 2018). Secondly, antibodies, themselves, can also be used to bind to cellular targets and act as effectors for many desired biochemical responses (Li and Zhu, 2010).

The exploitation of the naturally occurring properties of a biological molecule such as antibodies was a significant turning point of methodology in the pharmaceutical industry (Liu, 2014; Nelson et al., 2010). Like most significant changes, the introduction of biologics as medications was confronted with regulatory hurdles and skepticism (Baker and Harkonen, 1990; Petricciani, 1983; Sullman, 1990; Thorpe, 1993). Now that regulatory structures are in place for the use of antibody therapeutics in human subjects, they now constitute a sizeable portion of the revenue in the pharmaceutical industry. Since their introduction into the pharmaceutical industry in 1986 (Muromonab-CD3 OKT3, an immunosuppressant for organ recipients), antibody therapeutics produced nearly USD $90 billion in annual revenue in 2017 with a projected annual revenue of USD $125 billion by 2020 (CBR Pharma Insights, 2017; Ecker et al., 2015). Antibodies used as therapeutics and targeted delivery systems are currently the fastest growing biotechnology-derived compounds in human clinical trials, with no end of growth in the foreseeable future (Maggon, 2007). The success of antibody therapeutics embodies an excellent model for next-generation biologic (such as gene-editing nucleases) entry and expansion in the pharmaceutical sector.

Interfering RNA

Interfering RNA approaches can target and silence cellular RNAs (mRNAs) and also target and bind genetic elements directly to inhibit or activate transcription, each of which has therapeutic value. In the 1990s the discovery of RNA interference (RNAi) set the stage for another revolution in the pharmaceutical industry by promising for the first time, a shift from protein biomarker-targeting (defined by small molecules and antibodies), to downstream RNA biomarkers associated with human diseases. The transformation was the first to move closer to the genetic source of human diseases, the gene (DNA). While the power of gene silencing through RNAi held promise in the early days, there were many challenges that overwhelmed the technology such a delivery, stability, and intellectual property disputes (Aagaard and Rossi, 2007; Borel et al., 2014; Fougerolles De, 2008; Geldhof et al., 2007; Kim and Rossi, 2007; Tiemann and Rossi, 2009; Uprichard, 2005; Walchli and Sioud, 2008; Wang et al., 2008; Zhao et al., 2017). Fast forward nearly 20 years ahead to the present where many of these issues have been resolved, companies like Alnylam Pharmaceuticals (Cambridge, MA, USA) are flourishing with the market approval of the first RNA interfering therapeutic (Patisiran approved in 2017) for the treatment of Hereditary transthyretin (ATTR) amyloidosis (hATTR).

One of the positive outcomes related to the early stage issues mentioned previously that were associated with interfering RNA-based therapeutics is that vast improvement in delivery technologies such as stably targeted liponanoparticles (LNPs) and multi serotype inventions of viral delivery approaches such as lentivirus (LV) or adeno-associated virus (AAV) (Borel et al., 2014; Wang et al., 2008; Sarisozen et al., 2016; Shajari et al.,

2017; Shegokar et al., 2011). One can, therefore, predict that the CRISPR- Cas therapeutic sector will move ahead more rapidly by taking advantage of these earlier delivery innovations.

Stem Cell Therapy

Stem cell therapy is an indirect method to eliminate or compensate for the genetic deficiency by replacing or overriding existing diseased cells. Stem cell therapy is known to have promise in the treatment of many human diseases but there are unique challenges that exist with each method (each method being the cell type and source), including issues related to immunity, graft versus host rejection, the risks of teratoma formation or cancers, purification, expansion, maintenance, cost and reproducibility. In the early to mid-2000s several companies attempted to push **E**mbryonic **S**tem **C**ell (ESC) technology forward, but due to technical issues and ethical challenges most of these companies abandoned the technology and shifted their direction to more traditional pharmaceuticals such as small molecule compounds. For example, Geron Corporation (Menlo Park, CA, USA) started with a robust pipeline consisting of embryonic stem cell technologies for the treatment of multiple human diseases and injuries (such as Alzheimer's and spinal cord injury), only to divest the entire programme to Asterias Biotherapeutics (Fremont, CA, USA) and reposition its focus on an oligonucleotide-based compound for the treatment of myelofibrosis and myelodysplastic disorders (Hu, 2017; Ma, 2017) that inhibits telomerase activity (Imetelstat).

Other stem cell technologies such as **i**nduced **P**luripotent **S**tem **C**ells (iPSCs) will likely see a resurgence in the therapeutic arena due to the ease of use of CRISPR-Cas gene editing. CRISPR Cas tools may allow researchers to address some of the epigenetic challenges that have been associated with iPSCs in the past (Ji et al., 2016). Reignited interest in iPSC therapeutics will eventually rival alternate more established stem cell therapies such as **M**esenchymal **S**tem **C**ell (MSCs) drugs like Alofisel a TiGenix (Leuven, Belgium) and Takeda Pharmaceutical Company (Osaka, Osaka Prefecture, Japan) product that was recently approved in Europe. In general, the stem cell field will likely see a tsunami of innovation in the coming years.

CLASSICAL GENE EDITORS

Traditional gene editors referred to in this Chapter are the first players in the pharmaceutical sector to address diseases on the permanent genetic level directly. Gene editors such as ZFNs and TALENs are capable of targeting genes directly and altering the genetic sequence for positive therapeutic benefits. They can also be used to change genes in cellular and animal models to observe cause and effect relationships in disease state animals. Finally, they can be used to target genes to discover new biomarkers.

However, these classical gene editors are limited due to their large and bulky nature, making them challenging to deliver and resulting in low gene editing efficiency (compared to next generation editors) required for in vivo medications. Furthermore, it is difficult to introduce variability in their structures (due to the amino acid to nucleic acid contact sites - altering amino acids in the nuclease DNA binding domains requires multiprotein delivery to accomplish multi-DNA contact sites that occur in variable regions) (Fig. 8.1).

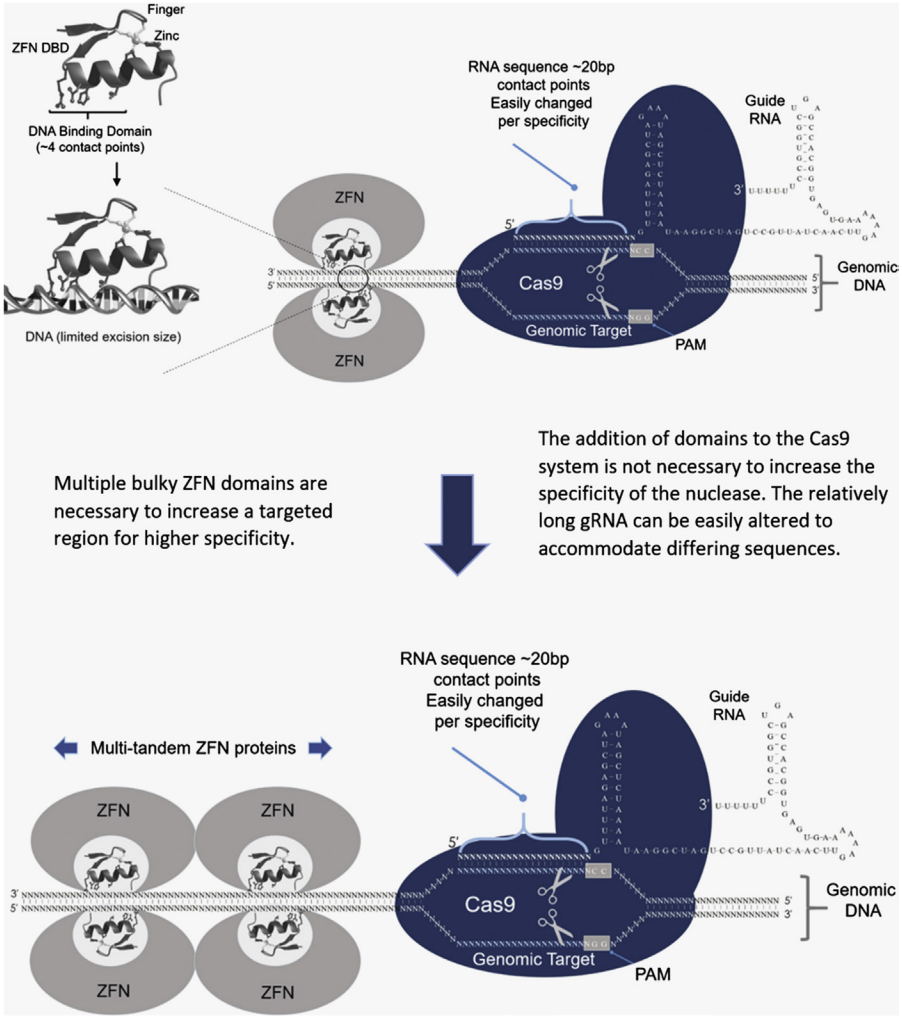

Figure 8.1 CRISPR Cas9 (and alternate Cas) nuclease(s) utilise an easily adaptable and versatile gRNA tract for targeting genetic sequences. This is unlike Zinc Finger Nucleases (ZFNs) that rely on amino acid to DNA base pair contacts, requiring multidomain engineering and bulky protein delivery.

Despite these limitations, one company, Sangamo Therapeutics (Richmond, CA, USA) is utilising ZFN and TALEN gene editing nucleases for the treatment of human genetic diseases such as haemophilia, sickle cell anaemia, Fabry disease, Beta-thalassaemia, Huntington's disease and other cancers (DiGiusto et al., 2016; Holt et al., 2010; Li et al., 2013b; Sharma et al., 2015; Tebas et al., 2014; Wang et al., 2014). Their approach circumvents most of the issues mentioned previously because they are utilising ex vivo gene editing techniques (see subsequently).

THE NEXT GENERATION GENE EDITOR

It is likely that gene editing approaches such as CRISPR-Cas nuclease-based therapeutics and diagnostics will have a far more significant long-term impact on the pharmaceutical industry compared to all the therapeutic strategies mentioned above. The primary advantages that CRISPR-Cas nuclease therapeutics hold over traditional small molecules, biologics (antibodies), interfering RNAs, and stem cells include: (1) CRISPR-Cas systems address disease at the genetic source, thereby circumventing the treatment approach and offering novel and exciting curative strategies; (2) they are simple, small, deliverable and cost-effective with which to work, opening the door to in vivo therapeutic strategies; and (3) the targeted design of guide RNAs (gRNAs) can be accomplished in a matter of weeks and at a fraction of the cost that plagues the discovery platforms associated with small molecules and antibodies. This latter point is particularly crucial because the elimination of these cumbersome discovery platforms will be a major driver for investment into fledgling companies across the industry, such that capital will likely be directed instead toward preclinical assay development resulting in a streamlined approach to human clinical trials. The technology allows years to be trimmed from the development process with savings in millions of dollars in the meantime (Fig. 8.2).

In this chapter, we analyse the next generation editors and the various types of modifications (described in the previous section) that can be adapted to them for drug discovery and therapeutic applications, and the impact of each on the biotechnology business landscape (opportunity, competition, and innovation).

Background

CRISPR is a microbial nuclease mechanism involved in defence against invading phages and plasmids (Horvath and Barrangou, 2010; Ishino et al., 2018; Sorek et al., 2008). The locus of CRISPR in microbial hosts contains a combination of CRISPR-associated (Cas) genes and non coding RNA elements capable of programming the specificity of the CRISPR-mediated nucleic acid cleavage (Haft et al., 2005; Jansen et al., 2002). CRISPR is based on the protein CRISPR-associated protein Cas, which bacteria and

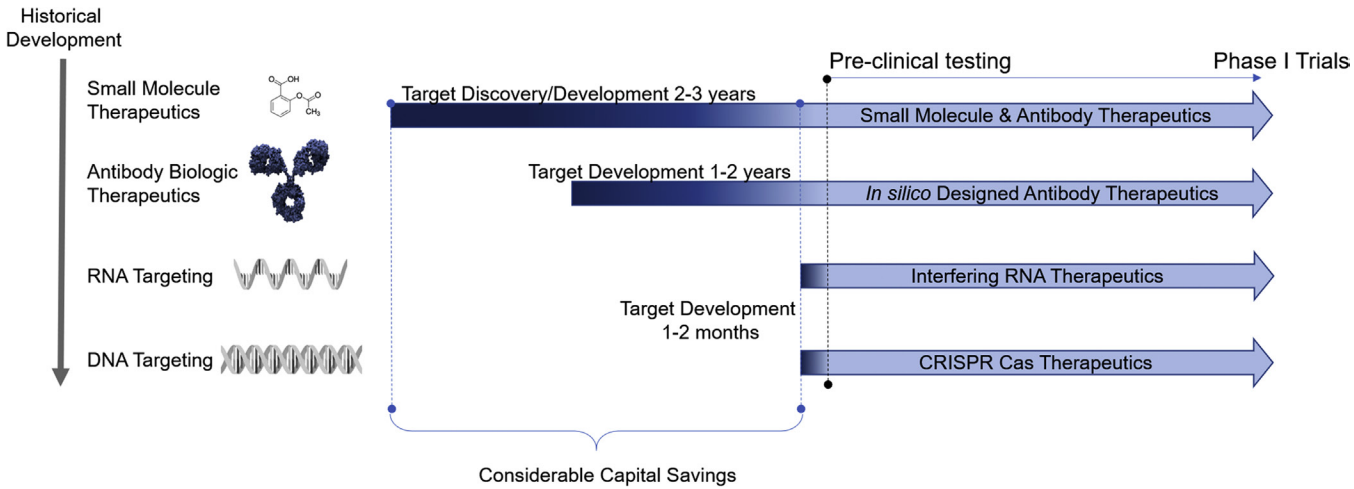

Figure 8.2 Next generation CRISPR gene-editing therapeutics address human diseases at the genetic source, thereby eliminating large amounts of time required for early discovery and development programmes. As a result, large amounts of capital can be instead diverted into preclinical testing and a stream-lined approach into human studies.

archaea wield as a tool to excise predatory bacteriophage (and viruses) DNA (Wiedenheft et al., 2009). CRISPR systems arm bacteria and archaea with a sequence-specific heritable 'adaptive immune system' that has a genetic memory of previous genetic invasions.

CRISPR

In 1987, a group from Osaka, Japan was the first to report clustered regularly interspaced palindromic repeat sequences (CRISPRs) (Ishino et al., 2018). They did this by showing five 29 bp repeats with 32 spacers close to the *iap* gene of *Escherichia coli*. Later in the following decade, reports as to the presence of CRISPR sequences were made for *Mycobacterium tuberculosis*, *Haloferax* spp. and *Archaeoglobus fulgidus*; however, these repeat sequences have not been observed in eukaryotic or viral DNA genomic sequences. Spacer sequences within the CRISPR locus are derived from both viral and plasmid origins (Shah et al., 2009). A group of researchers led by Francisco Mojica performed a comparative in silico study of the repetitive elements to investigate structure and sequence similarity (Díez-Villaseñor et al., 2013), and phylogenetic distribution (Richter 2013) showing that CRISPR sequences display a high degree of homology between phylogenetically distant species and a wide distribution in bacteria as well as archaea. Prior to these studies, it was also reported that CRISPR-Cas systems had been found in over 40% of bacterial and 80% of archaeal genome sequences available (Grissa et al., 2007). The CRISPR locus can be transcribed, demonstrating a nucleic acid component of the complex, whereas another element of the complex, CRISPR-associated (Cas) genes encode enzymes with active helicase and nuclease domains. The CRISPR- Cas loci consist of a CRISPR array of identical repeats that are intercalated with DNA targeting spacers that encode CRISPR RNA components and a Cas gene operon (which encodes Cas proteins) (Jansen et al., 2002; Bolotin et al., 2005; Mojica et al., 2005).

In the CRISPR array, repeats alternate with spacer sequences. In the arrays, the repeats are quintessentially identical in length and sequence, but variation occurs. Critically, the repeat at the array end is often shortened or digressed more from the canonical sequence. Repeats are usually between 23 and 47 bp in length and those with similar subtype share a concord sequence in type I–C, I–E, I–F, and II (Makarova et al., 2011a). Most of the repeat sequences display palindromes and a predicted stable secondary structure in the form of a stem-loop (Richter et al., 2012). However, there are repeats that are not palindromic and are unstructured. This variation in the ability to form secondary structures also has implications for the mechanism of pre-crRNA processing. In contrast, most of the spacer sequences in a genome are distinct. Many spacers have been paired with DNA sequences emanating from extrachromosomal origins, e.g., phage or plasmids and other transferable elements. A considerable number of spacers are

phage- or plasmid-derived, but the finite intensity of sequence data on these abundant and diverse elements leads to an underestimation (Stern et al., 2012). It has been discovered that some CRISPR-Cas systems also have spacers that pair with sequences elsewhere within its genome. Spacer length differs vaguely throughout an array by 1–2 nt and spacer length up to 72 bp have been publicised, but the size is often similar to that of the repeats in the same array (Richter et al., 2013a). The leader sequence, which is AT-rich, about 200–500 bp long and located upstream of the first repeat is a third of the CRISPR motif (Richter et al., 2013b), it is long and is needed for transcription of the array (Przybilski et al., 2011). The leader sequence is also essential for acquiring new spacers, and an individual genome can have one or more CRISPR array. CRISPR arrays appear to correlate with the degree of activity in spacer acquisition, with more extended arrays being further dynamic than shorter ones and arrays with degenerate repeat sequences (Richter et al., 2013b).

The CRISPR-Cas system is proposed to be an adaptive immune response system. This system documents antisense RNA as memory signals of previous invasions. Adaptive immunity is initiated by: (1) the introduction of short sequences of DNA into CRISPR array as a spacer sequence; (2) the pre-crRNA (precursor RNA) then undergoes transcription, maturation and generates crRNA which consists of a repeat portion (20 bps) and invader target spacer portion; and (3) the last stage includes crRNA directed cleavage of nucleic acid at complementary sites of spacer sequence of crRNA by Cas proteins. At present, there are three known distinct CRISPR-Cas systems: all three types of systems (I, II and III) utilise different molecular mechanisms for the recognition and cleavage of nucleic acids. crRNA-guided targeting via type I and III systems is performed by the large Cas protein complex, while the type II system only requires a single protein for recognition and cleavage of the DNA (Hille et al., 2018; Leon et al., 2018; Liu et al., 2018; Makarova et al., 2011b). Presently, CRISPR-Cas system type II is the most prominent one for genome editing (Wang and Quake, 2014). The major distinguishing feature of the three main CRISPR-Cas types is that the interference reaction of types I and III systems depends on a multiplex protein mechanism. The interference complicates the transfer of these systems to other organisms, and protein-modification of an individual Cas9 protein is more direct than the optimisation of Cascade, or Cmr/Csm complexes would be (Hale et al., 2012). However, the fact that type III systems do not require proto-spacer adjacent motif PAM sequences for interference should be beneficial for a more multifaceted editing incident (Marraffini and Sontheimer, 2010a,b). A major limitation of RNA-guided endonucleases (RGENs) genome editing tools is the protospacer adjacent motif (PAM) sequence, which is required for interference. Without the limiting PAM sequences for the type III interference, gene silencing can be achieved for a given target sequence. Nonetheless, specificity and the less sequence limitation is required to be at equilibrium, as the targeting of additional undesired sequences has been summarily avoided (Niewoehner and Jinek, 2017).

Cas9

The identification of 4 genes located on the CRISPR arrays (Richter 2013), which were coined CRISPR-associated (Cas) genes was instrumental towards the understanding of the role of CRISPR sequences. Bioinformatic analysis identified Cas9 as a multifunctional protein that encompasses two nuclease domains: HNH and RuvC-like. In *S. thermophilus*, Cas9 plays a role in combating viral invasion and is responsible for introducing double-stranded breaks in the DNA genomes of invading phages or viruses and plasmids, ultimately resulting in the in vivo targeting of plasmids and phages in bacteria. The Cas proteins are critical to the differentiation between the primary CRISPR-Cas types, and likewise, their subtypes. The two major classes of Cas proteins are easily distinguishable; the first class includes the core Cas proteins found in all CRISPR-Cas types. Cas1 and Cas2 are found in Cas operon of the three major types, and Cas1 is universally accepted as the biomarker for CRISPR-Cas systems. Cas3, Cas9, and Cas10 serve as signature proteins for type I, type II, and type III, respectively (Makarova et al., 2011a,b). The second class comprises of proteins only found in the gene clusters of a specific subtype (Richter 2013). The subtype-specific proteins of some CRISPR-Cas systems form a complex involved in targeting and interference, which for the type I-E system is referred to as CRISPR-associated complex for antiviral defence (Cascade) (Nam et al., 2012a). The systems are complexed because some bacteria have multiple CRISPR-Cas subtypes, and each has a series of CRISPR arrays which functions with a suitable Cas cluster (Nam et al., 2012b).

CRISPR Systems as Genome Editing Tools

The application of the various CRISPR-Cas systems as genetic tools has a bright future. Before the discovery of Cas protein functions, CRISPR sequence diversity was employed mostly to identify similar bacterial strains. This genotyping method is referred to spoligotyping-spacer oligonucleotide typing (Richter et al., 2013b). The examination of various Cas protein activities established them as diverse components for genetic tool development. A pertinent example is Cas6f, which is used in predictable gene expression. Cas6f cleavage site sequence of a CRISPR repeat can be fused to a gene of interest (Richter et al., 2013a). Cas6f processing of the cleavage site at the mRNA level can be utilised to detach untranslated regions (UTRs), ribosome binding sites and other regulatory elements. Therefore, transcript blockade can be minimised, which often leads to a more predictable gene expression pattern (Qi et al., 2012).

Also, the high substrate affinity of Cas6f was utilised in creating a specific RNA-binding 'lure' protein. This protein can be used in high-throughput RNA affinity purification protocols to isolate RNA molecules with a 16-nucleotide hairpin sequence derived from the 3′-terminal crRNA repeat tag (Lee et al., 2013). The analysis of Cas protein interference complexes revealed their vast potential for the development of

genetic tools that are required to provide specific DNA or RNA targeting (Richter et al., 2013b). A major component of this complex is Cas9. Integration of a nuclease-inactive mutant of Cas9 and a sequence-specific crRNA can be employed for targeted DNA recognition to interfere with transcriptional elongation, transcription factor binding or RNA polymerase. This gene silencing activity was coined CRISPRi (Qi et al., 2012). Subsequently, others reported the utilisation of Cas9/crRNA complexes for genome editing in different organisms, Cas9 interference experiments indicated that the fusion product of crRNA and tracrRNA has similar efficiency as the RNase III-processed crRNA:tracrRNA duplex (Jinek et al., 2012). These genome editing approaches use Cas9 together with a specific small guide RNA (sgRNA), which was designed to resemble the fused crRNA/tracrRNA sequence (Cong et al., 2013). The resulting ribonucleoproteins are termed RNA-guided endonucleases (RGENs) and were shown to target single genes or numerous genes, permitting competent and site-specific editing of the target sequence (Gratz et al., 2013a,b). The sgRNA sequence determines the targeting reaction specificity; Cas9 can be retargeted efficiently using various sgRNA sequences. It was shown further (Wang and Quake, 2014) that the utilisation of several sgRNAs in one reaction permits multifold editing of up to 5 genes. CRISPR genome editing methods enable sgRNA cas9 systems to create double-strand breaks of the targeted sequence; it can be restored by homologous recombination and non homologous end joining. In homologous recombination, double strand breaks are repaired, as the wildtype target sequence allele serves as a donor template. However, the non homologous end joining is a repair mechanism, illegitimate recombination that is prone to errors. NHEJ can give rise to indels (insertions/deletions) that often results in target sequence mutations (Zhao et al., 2017; Wang et al., 2016). A modified donor DNA, which serves as a template for homologous recombination, is employed to enhance the yield of the edited sequences.

CRISPR Cas9 Variants, Adaptations, and Purposes as a Tool for Drug Discovery

Beginning with the advent of Cas9, many scientists have developed and characterised alternate versions of the Cas9 protein with functions that allow it to be used for multiple purposes, from diagnostics (Uppada et al., 2018) to genetic and biomarker drug discovery in the subsequent sections is a discussion of some of these applications.

CRISPR-Based Transcriptional Activation and Repression

The addition of transcriptional transactivation or repression/inhibition domains to create a fusion chimera with the Cas9 (CRISPRa and CRISPRi, respectively) nuclease allows for the direct observation of gene regulation in mammalian systems without the need for reporter constructs (La Russa and Qi, 2015). In these applications, the nuclease

activity of Cas9 is deactivated (forming dCas9), allowing the protein to associate but not nick the target DNA. This application enables dCas9 associated with a designed and targeted gRNA to bind to a promoter region of a gene, followed by the transactivation domain activating the specifically targeted gene (Gilbert et al., 2014; Gilbert et al., 2013; Konermann et al., 2015; Perez-Pinera et al., 2013). This level of gene regulation and study is not novel in more simplified experimental model organisms such as yeast (e.g., the yeast two-hybrid system), but can now be adapted very efficiently, rapidly and effectively to any mammalian cell type (Gilbert et al., 2013).

The activation/repression of validated and targeted genes in this manner will provide more efficient and cost-effective options for examining insights into gene function. The controlled manipulation of the expression levels of genes thought to be involved in different types of cancers will allow researchers to study the upstream genetic targets of proteins that are believed to be oncogenic. This approach will provide the community with a trove of new data giving researchers the tools to understand better the intricate interactions between oncogenes and wildtype proteins and their effects on the circuitry of the cell (Kasap et al., 2014; Shi et al., 2015). Already, this field of study is making enormous strides with improvements in pioneering chimeric technologies. For example, the VP64 transcriptional activation domain linked to dCas9 was one of the first chimeric proteins used to study transcriptional activation models in mammalian cells (Ji et al., 2016; Maeder et al., 2013). This system has recently been improved by combining in a specific order, multiple activator domains including VP64, p65, and Rta to create a VPR tandem activation complex that is capable of being targeted to specific genes and increase their expression from ~5 to ~300 fold (Chavez et al., 2015; Guo et al., 2017). The easy targeting, and delivery of these types of systems in mammalian cells, combined with both highly sensitive and increased expression levels of targeted genes is going to lead to a far more detailed, corrected and remapping of cell signaling pathways.

The effects of traditional therapeutics on the expression levels of targeted genes can also be studied using CRISPRa/CRISPRi techniques. Importantly, the approach is already being used to reveal the complex genetic networks that regulate drug resistance pathways (Jost et al., 2017; Kurata et al., 2018). A clear understanding of these networks will allow future researchers to develop new molecules that could counter drug resistance pathways or use CRISPR-Cas gene editing to create preventative mutations in the network that lead to drug resistance.

CRISPRa/CRISPRi systems are currently being used to explore the effects of expression levels on cell surface receptors and to identify new biomarkers (Gilbert et al., 2014; Gebre et al., 2018; Kampmann, 2018; le Sage et al., 2017; Wang et al., 2018; Zhang et al., 2018). These types of approaches will have a direct impact on human infectious diseases. For example, Gilead Sciences (Foster City, CA, USA) recently awarded $7.5 million in academic grants, in part, to use CRISPR systems (may or may not be

CRISPRa/CRISPRi approaches, likely but data unavailable) to unlock cell surface markers related to latent human immunodeficiency virus (HIV) pools (Crunkhorn, 2017; Park et al., 2017; Tsui et al., 2017). Once a cell surface marker is defined, CRISPR Cas engineered Chimeric Antigen Receptors on T cells (CAR-T) or modified T cells could then be developed (using ex vivo techniques from patient samples) to seek out the newly discovered HIV latency-associated cell surface biomarker and tag the cell for destruction. CRISPRa/CRISPRi will become an increasingly more unique and vital methodology, especially in the context of biomarker target discovery and the development of CAR-T. CAR-T therapeutics is one of the hottest (based on the enormous amount of intellectual property filings in the last 3 years alone) and most exciting developments in the pharmaceutical industry, and CRISPR Cas gene editing is playing a central role in its development (Androulla and Lefkothea, 2018; Liu et al., 2017; No Authors Listed, 2017). Generally speaking, CRISPR Cas gene-editing techniques have two hands in the CAR-T space (Fig. 8.3). Firstly, CRISPRa/CRISPRi can be used to define better or unlock new cell surface receptors and other biomarkers related to a disease state (infectious or genetic) as mentioned in the previous section (HIV as an

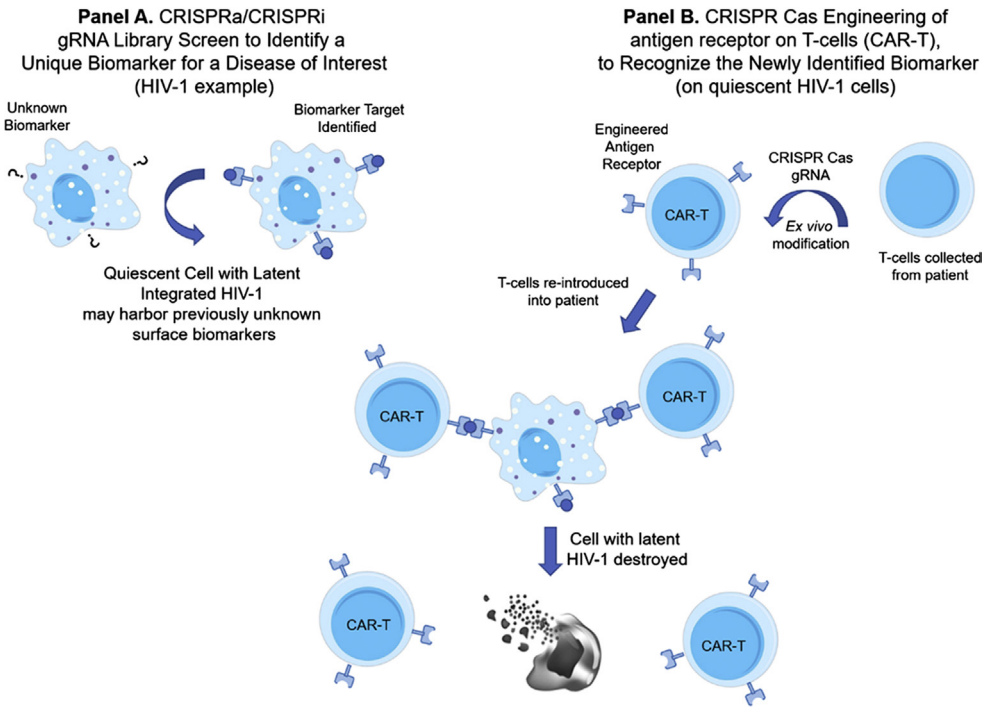

Figure 8.3 CRISPR Cas systems can be used in tandem by discovering unique and specific biomarkers, coupled with targeted cellular therapeutics such as CAR-T.

example, cf. Fig. 8.3, Panel A). Secondly, once defined, these cell surface markers can be targeted by engineered CAR-T cells. The CAR-T cells would be genetically constructed in an ex vivo manner (Panel B) thereby becoming a cellular-based drug, a 'living drug', capable of very specific recognition of the cell surface marker of choice once reintroduced into the patient. Currently, there are multiple companies involved to some degree with developing CAR-T therapeutics. These include Kite Pharma (Los Angeles, CA, USA) (now Gilead), Juno Therapeutics (Seattle, WA, USA) (now Celgene), Celgene (Summit, NJ, USA), Cellectis, and Vertex. Although CAR-T therapeutics hold tremendous promise to treat human diseases, the technology is in its infancy and CRISPR Cas gene editing, especially its use as a gene study and discovery tool, will play a major role in its development by better defining highly unique cell surface markers for specific targeting. From a business perspective, the use of CRISPRa/CRISPRi gene expression methods will open entirely new opportunities for companies in the industry to develop new and safer approaches for all types of therapeutics by better defining biochemical interactions related to disease states and the effect of drugs on those states.

Conjugation of Deaminase Enzymes to Cas9

Fusion chimera constructs of Cas9 and an exogenous deaminase are used to ensure high efficiencies and the precise introduction of single base mutations at selected target loci (Kim et al., 2017a; Komor et al., 2016). Many cancers can be traced back to a single mutation in a critical tumor suppressor gene, such as the BRCA1, BRCA2 (Bishop, 1994; Casey et al., 1993) and p53 (DeLeo et al., 1979; Rodrigues et al., 1990) tumor suppressors. This method allows for the correction of that mutation within the model organism of choice. It is likely that additional modifications to Cas nucleases will be made in this respect (Li et al., 2018), so that any base can be corrected in a gene, from single to double to multiple mutation replacements, to also be used to insert bases to restore gene function from a crippling frameshift mutation. This high precision editing could be the wave of the future for many Cas9 nuclease-based therapeutics, eliminating the need for multiple base pair corrections thereby resulting in a 'cleaner' outcome with less chance of inadvertently introducing another mutation or frameshift. Highly precise Cas9 therapeutics in the future will likely contain a deaminase (or similar) domain fused to a platinum Cas9 nuclease (described in the subsequent section).

The Conjugation of Fluorescent Proteins to Cas Nucleases

In many instances of Cas-based therapeutics, the ability to visualise the nuclease:DNA complex is essential to determine the number of loci with which the nuclease is interacting. This monitoring can be accomplished by creating a fusion of one of the many fluorescent proteins and Cas nucleases. This fusion protein allows for the visual tracking of Cas-targeted nucleases to the genes of interest in both cell culture, animal models and possibly humans (Chen et al., 2013). Currently this approach can be used for ex vivo

visualisation (Anton et al., 2014). The fusion protein also allows for the monitoring of potential off-target interactions, should any exist. Finally, expression of this type of chimera permits the assessment of the amount of fusion protein produced in cells. There are indeed more applications for fluorescently tagged Cas nucleases than mentioned here, some of these applications will undoubtedly lead to new scientific directions for industry and academia.

The fluorescent or radioactive tagged Cas nucleases can also be used in concert with other CRISPR-associated techniques (Khoshnejad et al., 2018). For example, the combination of fluorescently labelled Cas9 with existing (or CRISPR nuclease-based) biomarker assays has the potential to create a substantial correlation between the onset of disease and genetic mutations using a diagnostic approach. Improvements in Cas9 off-targeting will undoubtedly increase the accuracy, precision, specificity and robustness of these types of diagnostic tools (Uppada et al., 2018).

CHALLENGES ASSOCIATED WITH CRISPR-CAS9 AS A GENE EDITING THERAPEUTIC (IN VIVO AND EX VIVO PERSPECTIVES)

Although the prognosis for CRISPR-Cas techniques and applications look promising, there are indeed technical challenges that still to this date require addressing. Nuclease delivery efficiency, off-target effects on nontargeted genes, gene editing escape mechanisms, the introduction of NHEJ-derived mutations, and immunity against sa/sp Cas9 due to previous infections from *Staphilococci* and *Streptococci* are the current challenges faced by the CRISPR Cas9 gene editing community, and more unforeseen technical issues will likely arise (Wang et al., 2016; Charlesworth et al., 2018). New delivery and expression approaches may be needed to overcome some or all of these challenges.

Off-Target Effects

Cas9 is known to cause unintended off-target effects that nevertheless can be minimised by the mutations discussed in the previous section. For human genetic diseases, this can be a challenge because it may be difficult to find highly conserved regions at the targeted deficient gene locus that correspond to the PAM sequence of Cas9. It is to be noted that there are altered versions of Cas9 now available that do not require a PAM sequence, as well as next-generation Cas nucleases that each have different PAM recognition properties so that this limitation may be antiquated (Burstein et al., 2017; Hu et al., 2018; Karvelis et al., 2017; Kleinstiver et al., 2015). For ex vivo therapeutics of the Cas9 nuclease, off-targeting effects are less of a problem because the altered cells can be thoroughly sequenced outside of the body and checked for off-target effects before reintroduction into the patient. For in vivo therapeutics of the Cas9 nuclease, off-targeting remains a challenge primarily when targeting human genes. For these therapeutics, the Cas9 off-target mutants would be favourable. Mutational analysis was performed to alleviate or minimise the

potential off-target effects on Cas9 (Kleinstiver et al., 2016), and similar analyses can be applied to additional Cas nucleases. Multiple Cas9 proteins now exist (termed the Platinum series by Editas Medicines (Cambridge MA, USA)) that contain backbone mutations allowing the Cas9 to retain its activity while reducing or abolishing the off-target effects.

For many exogenous genes that are targeted, such as viral genes, this becomes much less of an issue because viral genes have many highly conserved and unique genetic segments that coordinate with PAM sequences, but that do not cross-react with human sequences (Kaminski et al., 2016). Despite the challenges, there are in place off-target monitoring technologies such as CIRCLE-Seq (Tsai et al., 2017) and ultra-deep sequencing protocols to pinpoint local variations and potentially correct them (Koo et al., 2015).

NHEJ Introduction of Mutations

It is essential to use more than one guide RNA (gRNA) if the objective is to eliminate large portions of a gene to avoid viral escape mechanisms. This problem exists for in vivo and ex vivo therapeutics (Wang et al., 2016). By using two or more guide RNAs, critical elements of the viral genome can be 'excised' and therefore, NHEJ escape is taken off the table (Kaminski et al., 2016; Hu et al., 2014; White et al., 2016; Yin et al., 2017). (Notably for infectious diseases, this has significant implications as mutant strains of the virus can result from NHEJ).

CRISPR CAS THERAPEUTIC FORMULATIONS AND MODES OF DELIVERY IN THE CONTEXT OF THERAPEUTICS

CRISPR Cas therapeutics will have two mainstream approaches to improve or cure disease; (1) ex vivo modification of disease-targeted and relevant cells that will themselves be delivered as either a replacement therapeutic or a living drug, and (2) In vivo delivery of Cas nucleases with gRNA(s) in the form of (i) a DNA expression vector, (ii) an mRNA combined with a targeted gRNA(s) oligonucleotide, or (iii) a combination of protein and a targeted gRNA(s) oligonucleotide (Ul Ain et al., 2015).

1. The ex vivo CRISPR Cas approach to altering cells involves multiple and costly technicalities that are described subsequently in paragraph 22. The initial step in the ex vivo approach is identifying the cells that harbour a target that is associated with a state of disease (a unique cell surface marker for example) within the patient. Once identified, these cells can be collected then treated (via transfection or electroporation) with CRISPR Cas nucleases plus targeted gRNA(s), that when expressed within the cell will repair the genetic abnormality that is related to the disease. Once the abnormality is corrected, the cells would then be reintroduced into the patient through intravenous or tissue-injectable methods. Although this approach is the most direct method for replacement therapy, residual cells (diseased cells that remain in the body) would likely recover (recurrence), thereby requiring subsequent and continual suppressive therapies. Another,

yet more robust ex vivo approach, is to modify patient cells (or allogeneic cells) collected from the body, so that they recognise cells in a disease state within the patient to destroy them (Androulla and Lefkothea, 2018; No Authors Listed, 2017). In this method, the patient's healthy cells (or donor cells) would be collected, purified, then altered using CRISPR Cas plus gRNA(s) that target relevant genes in the cell, thereby turning them into living drugs. The cells would then be reintroduced back into the patient through intravenous or tissue-specific injectable methods. These methods are safer because the cells that are modified outside the patient's body can be rigorously tested against issues such as genetic off-targeting, chromosomal aberrations that may result from NHEJ, and phenotypic changes. Further, any immunogenicity issues that might be associated with Cas9 would be avoided entirely (more subsequently).

2. In vivo treatment using a Cas nuclease(s) plus gRNA(s) that are targeted to correct a genetic abnormality related to a given disease is the holy grail of the gene-editing therapy field. In this approach, the Cas nuclease and gRNA(s) are delivered either as a DNA expression vector, RNA or protein/gRNA(s) combination. When this method is used, very careful design of the gRNA(s) is required to minimise or avoid genetic off-target effects that may cause irreversible damage to the patient's genome. Further, depending on the disease, delivery to specific cells or tissues represents a challenge. Cas nucleases and gRNA(s) can be delivered multiple ways by using liponanoparticles (LNPs) (Finn et al., 2018), adeno-associated viruses (AAVs) (Senís et al., 2014), nanovesicles (NVs), exosomes (Kim et al., 2017b), exosome/LNP hybrids (Lin et al., 2018) and clews (Sun et al., 2015), to name some of the main types. Moreover, each of these delivery systems can be targeted to specific cells and tissues by conjugating target-complementary ligands such as antibodies, engineered receptors or carbohydrates.

Despite the large repertoire of delivery systems available, some of the challenges that exist among them include toxicity, immunogenicity, cellular and tissue accessibility, biochemical packaging, manufacturing (scalability), target cross-reactivity (specificity), and stability (half-life). For the most part, the treatment of human genetic disease using in vivo methods will require further delivery development before it becomes mainstream as a product. Over the next decade, there will likely be a renaissance of delivery methods based on the improvements of existing methods, as well as novel approaches such a nanorobotics (NR) (Henderson, 2007), thereby opening the door to considerable business opportunities.

In the meantime, non hereditary diseases such as those caused by infectious agents including bacteria, fungus, parasites and viruses embody 'low hanging fruit' in the in vivo CRISPR Cas/gRNA therapeutic arena. Most of these therapeutics can be delivered with existing LNP or AAV technologies. AAV delivery systems have been around for years but most of the therapeutic payloads related to gene-editing therapeutics (ZFNs and TALENs) have been too large to package into the vector, so products related to the system have never truly flourished. Now, with the discovery of much smaller gene editors such as saCas9, CasX and CasY that can easily fit into the vector deliverable (Senís et al., 2014),

AAV systems are seeing an enormous surge in business opportunities, reflected by considerable growth in companies like RegenxBio (Rockville, MD, USA).

Immunity Against Staphylococcal and Streptococcal Cas9

Although it has always been suspected that natural immunity against these forms of Cas9 may exist from *Streptococci* or *Staphylococci* infections earlier in an individual's life, this notion was never examined until 2018 (Charlesworth et al., 2018). The study found that up to 79% of the individuals tested were positive for streptococcal or staphylococcal antibodies, and 45% of the tested individuals had adaptive immunity. Although the study sample was relatively small compared to real population dynamics, there may be severe implications, including for example the neutralisation by the humoral immune response, or T cell mediated death by the adaptive immune response, for the use of these forms of Cas9. However, because the genetic modifications are performed outside the body and away from the immune response, this challenge can be avoided or minimalised as all ex vivo modifications of genes in cells will not result in Cas9 protein being present in the final cell-based therapy. Currently, this is one of the major arguments against in vivo therapeutics, a viewpoint that is further explored in subsequent paragraphs.

Many businesses are currently geared toward ex vivo modifications, because the risk is lower and the pathway forward with regulatory agencies is already established, due to previous gene-editing methods using traditional editors; all of which were conducted ex vivo, by using tools such as ZFNs, that have been in human clinical trials for some time with companies such as Sangamo Therapeutics. The main issue, however, with ex vivo therapeutics, is their very high costs, which can be ascribed to the multistep process outlined in Immunity Against Staphylococcal and Streptococcal Cas9 section.

What Drives the High Cost of These Therapeutic Interventions?

There are several reasons for the high cost of treatment of gene-edited cell-based therapies, some of which are listed as under:

1. Cell and tissue collection from the appropriate bodily organ of the patient
2. Cell selection of the diseased cell or the cell that will be modified to attack the disease, such as is the case with CAR-T approaches
3. The enrichment of the cell from other cell types present in the organ
4. Genetic modification of the cellular genome using the Cas9 nuclease system
5. Enrichment of the modified cells in a cell or tissue culture setting
6. Examination of the cells to assess the values of safety-related biological parameters. These include, but are not limited to: whole genome sequencing, surface receptor alterations and viability
7. Injection back into the patient. In addition, to this process, some of the modified cells need to be stored for future treatment
8. Cell storage and management costs

For the above reasons, genetically altered ex vivo therapeutics is extremely expensive, reaching between $400k to $1M per treatment (Mullen, 2018). From a business perspective, the reduction of these costs would require one to capture economies of scale and scope via specialised infrastructures that currently do not exist. There is an extraordinary opportunity for a centralised business model to address such an infrastructure vacuum. Nonetheless, costs of the therapeutics are bound to decrease significantly as more therapeutic doses are produced thus generating economies of learning. The speed at which the new products become available poses a major problem for regulatory agencies; what is more, the public increasingly scrutinise the justification for what is bound to be perceived as much higher drug prices, thereby creating market-driven pressures that will threaten to cut profits. A key here is to balance the prices of highly innovative therapies with the values that these therapies ultimately deliver to the patients and the payers.

The golden therapeutic approach lies however, in in vivo alterations. Directly delivering into patients a product that will carry out in vivo the needed genetic modifications to treat a particular disease will eliminate the need for a complex personalised engineered cell-based therapy manufacturing infrastructure. There are, however, some challenges that need addressing before this can become a reality. Notably, in vivo approaches are hindered by potential problems due to immunogenicity, off-target effects, and other short-comings, i.e., those parameters that can be tested in ex vivo settings. Regarding immunogenicity, in vivo approaches can be tailored to potentially minimise humoral or adaptive immunity. Humoral immunity may be minimised on the other hand by the delivery of DNA vector-based therapeutics to the cell. In the case of Cas9 (*of Staphylococcus* or *Streptococcus origin*), the protein would be neutralised rapidly and may trigger a robust humoral response from preexisting exposure to these bacteria (Charlesworth et al., 2018). In the case of new Cas editors, the presence of nuclease outside the cell may trigger a primary humoral reaction and hinder any future treatment with any such nuclease. Therefore, the notion that humoral immunity can be avoided by using alternate Cas nucleases may only be a temporary fix. From a business perspective, companies that hold multiple Cas nuclease alternatives in their in vivo disease treatment repertoire may be the ones that flourish in the long term.

Example 1 – a patient with HIV may be efficiently treated with Cas9 for a single treatment. Many years later the same patient may become reinfected with HIV and Cas9 may not work so that an alternative nuclease would be necessary.

Example 2 – a patient with lymphoma may be treated in vivo with Cas9. Despite remission for this particular cancer, the same patient may later be inflicted with another cancer and require an alternate Cas nuclease, and so on.

The existing immunogenicity from previous staphylococcal or streptococcal infections may limit Cas9 therapeutics to a single treatment. Other Cas nucleases have been discovered in bacteria that typically do not infect humans, and therefore it is unlikely that preexisting immunity exists in the bulk of the population. Isolation from a bacterial source means that these nucleases could be used for multiple treatments before a 'therapeutic-derived immunity' response develops.

CRISPR-Cas Nucleases Therapeutics and the Progress From Concept to Market

In addition to Cas9, the CRISPR Cas nuclease tool box is growing due to the invention of Cas9 mutants, the conjugation of enzyme domains to Cas9 (and potentially other Cas nucleases) that carryout various functions, and the discovery of new Cas nucleases such as Cpf1, CasY, and CasX. As the number of tools grows, diversity in the market will expand based on the different approaches to treat therapeutics and exponentially high number of applications for which they can be adapted. Each of these tools will have their own unique challenges that will contribute to niche market growth and defining all of them is beyond the scope of this chapter. Below is a brief outline of how the current intellectual property challenges associated with Cas9 (also beyond the scope of this chapter) may become nullified due to the discovery of new editors. Newly discovered Cas editor tools do not have the baggage of lawsuits (at least to the degree associated with Cas9), and therefore venture capital groups will be less reluctant to invest in start-up companies. This, in combination with streamlined development programmes (summarised subsequently in the context of therapeutics) and a shorter time to market (compared to traditional medicines) will lead to an avalanche of new market-driven opportunities.

The CRISPR-Cas Collection

The current focus is on the editor, Cas9, and the recent variations of this nuclease. This focus is due to its initial discovery and full adaption across many research settings in a short period of time, coupled with a media-driven promise of a new era in medicine. Initially, most scientists realised that additional editors or editor activities would be present in different bacteria and archaea, as well as in simpler eukaryotes. This assertion is intuitive due to parallels that can be drawn to the endonucleases discovered and developed in the 1980s and 1990s, where companies were competing over the restriction enzymes *Bam*H1, *Eco*RV, and *Hin*dIII. New CRISPR sequences are continuously being uncovered with the promise that more will come. With the identification of hundreds of endonucleases, the intellectual battles around the three original systems have become moot (Sherkow, 2018). The promise of newly discovered CRISPR complexes is setting the stage for companies that will avoid much of the intellectual property issues that, until recently, have been associated with CRISPR-Cas9. The characteristics of these new Cas nucleases are unknown mainly due to their recent identification and as such, have not been tested to the extent of Cas9. These new nucleases are small and deliverable using methods such as adenoviral vectors. Also, each has different PAM recognition capabilities. As previously discussed, these capabilities may be advantageous and could expose previous Cas9 inaccessible sequences leading to new biomarker target discovery and other Cas-dependent classes of drugs derived from biomarker discovery.

Also, these discoveries will offer a broader range of therapeutics and accessibility to alternative PAM sequence targets of genes associated with the disease. However, unlike the endonucleases the number of which range in the hundreds, it is likely that far fewer CRISPR nucleases will be found due to the low number of variant types per bacterium, homologous forms in different species, and divergent Cas nucleases that exist in bacterial species that are difficult to culture. The limited number of CRISPRs, however, will not negatively impact drug discovery, because new CRISPRs have the potential of having different properties and PAM recognition sequences, thus allowing for more breadth and accessibility to genetic sequences. The limited number will, however, impact the number of treatments over a long-term among the population and may give rise to 'CRISPR resistance' that would be tantamount to the 'antibiotic resistance' that we currently see for bacterial disease treatments.

PERSPECTIVES: FROM CONCEPT TO MARKET

CRISPR Cas systems have the versatility and ease of use to change the way one treats numerous human diseases. The technology's versatility lies in its use across many aspects of drug discovery, diagnostics, therapeutic creation through genetic alterations (CAR-T therapies), stem cell and ex vivo patient cell manipulation, and as an in vivo therapeutic itself. Its ease of use lies in its easily adjustable targeting system that revolves around its gRNA property, giving the designer access to virtually any genetic sequence for cancers (Chen et al., 2016; Liu and Zhao, 2018), infectious diseases and inherited diseases.

The design of testable gRNAs for any given target sequence currently takes approximately 1–2 months. However, as more biomarker's related to critical properties of a given disease (such as the replication cycle for a virus, antibody resistance markers in bacteria, oncogenes, etc.) are identified (using CRISPR ironically) and computing platforms improve it, it is likely that this time gap will be narrowed to within a few hours in the near future, if one uses as a parallel the dramatic increase in sequencing speed and throughput achieved over the last decade. This will allow researchers to jump immediately into cellular models followed rapidly by in vivo animal studies and eventually to the clinic. Importantly, as more Cas-based therapeutics enter the clinic, it is likely that clinical trials will become more streamlined, attributed to the consistency, deliverability and predictability of Cas nuclease performance. Once approved for market, depending on the nature of the therapeutic, there will of course be manufacturing and distribution challenges.

Should CRISPR Cas-based therapeutics become the norm for treating most human diseases (after both the current and unpredicted challenges are resolved), one will truly witness the 'gene-editing revolution' kick into gear in the pharmaceutical industry. Many companies, in addition, to the existing CRIPSR Companies listed in Table 8.1, will form and flourish as the technology expands and new uses are discovered. In such a scenario, many patients will benefit from this new era in medicine.

Table 8.1 A List of the Current Companies in the CRISPR Space That Are Developing CRISPR Cas-based Therapeutics.

Company	Symbol	Location	Founder(s)	Pipeline/Disease Focus	Editing MOA	Delivery Mode	Nuclease
EDITAS Medical	EDIT	Cambridge, MA	Feng Zhang, George Church, J. Keith Joung, David R. Liu	Leber's Congenital Amaurosis (LCA)	Disruption	AAV Local Injection	Cas9
				Duchenne Muscular Dystrophy (DMD)	Disruption/ Repair	AAV or LNP	Cas9
				Usher Syndrome 2a, HSV-1	Disruption/ Repair	AAV Local Injection	Cas9
				β-Thalassaemia, Sickle Cell Disease	Disruption/ Repair	RNP ex vivo	Cas9
				Cystic Fibrosis	Disruption/ Repair	AAV or LNP	Cas9
				Alpha 1-Anti-trypsin Deficiency	Disruption/ Repair	AAV or LNP	Cas9
				CAR-T Cell Line Generation	Disruption	RNP ex vivo	Cas9
Intellia Therapeutics	NTLA	Cambridge, MA	Rodolphe Barrangou, Nessan Bermingham, Jennifer Doudna, Rachel Haurwitz, Luciano Marraffini, Andy May, Derrick Rossi, Erik Sontheimer	Transthyretin Amyloidosis (ATTR)	Disruption	LNP	Cas9
				Hepatitis B Virus (HBV)	Disruption	LNP	Cas9
				Alpha-1 Antitrypsin Deficiency (AATD)	Disruption/ Repair	LNP	Cas9
				Primary Hyperoxaluria (PH1)	Disruption/ Repair/Insertion	LNP	Cas9
				Hematopoietic Stem Cells (HSC)	Disruption/ Repair	electroporation ex vivo	Cas9
				Chimeric Antigen Receptor T Cell (CAR T)	Disruption/ Repair/Insertion	electroporation ex vivo	Cas9

CRISPR Therapeutics AG	CRSP	Cambridge, MA	Rodger Novak, Emmanuelle Charpentier, Shaun Foy, Chad Cowan, Daniel Anderson, Craig Mello, Matthew Porteus	β-Thalassaemia	Disruption	ex vivo	Cas9
				Sickle Cell Disease	Disruption	ex vivo	Cas9
				Hurler's Syndrome (MPS-1)	Disruption/ Repair	ex vivo	Cas9
				Severe Combined Immunodeficiency (SCID)	Disruption/ Repair	ex vivo	Cas9
				CD-19-Positive Malignancies	Disruption &/or Repair	ex vivo	Cas9
				Anti-BCMA Allogenic CAR-T	Disruption &/or Repair	ex vivo	Cas9
				Anti-CD70 Allogenic CAR-T	Disruption &/or Repair	ex vivo	Cas9
				Glycogen Storage Disease la (GSD la)	Disruption/ Repair	in vivo	Cas9
				Haemophilia	Disruption/ Repair	in vivo	Cas9
				Duchenne Muscular Dystrophy (DMD)	Disruption	in vivo	Cas9
				Cystic Fibrosis (CF)	Disruption/ Repair	in vivo	Cas9
Excision Bio Therapeutics	Private	Philadelphia, PA	Kamel Khalili, Thomas Malcolm	HIV-1	Excision	AAV or Lenti Virus	saCas9
				JC Virus (PML)	Excision	AAV	saCas9
				HSV-1	Excision	AAV	saCas9
				HSV-2	Excision	AAV	CasY
				HBV	Excision	LNP	CasX
				HTLV-1	Excision	AAV	CasX

REFERENCES

Aagaard L, Rossi JJ. RNAi therapeutics: principles, prospects and challenges. Adv Drug Deliv Rev March 30, 2007;59(2–3):75–86.

Adli M. The CRISPR tool kit for genome editing and beyond. Nat Commun May 15, 2018;9(1):1911.

Akhmetov A, Laurent JM, Gollihar J, Gardner EC, Garge RK, Ellington AD, Kachroo AH, Marcotte EM. Single-step precision genome editing in yeast using CRISPR-Cas9. Bio Protoc March 20, 2018;8(6).

Androulla MN, Lefkothea PC. CAR t-cell therapy: a new era in cancer immunotherapy. Curr Pharm Biotechnol April 17, 2018.

Anton T, Bultmann S, Leonhardt H, Markaki Y. Visualization of specific DNA sequences in living mouse embryonic stem cells with a programmable fluorescent CRISPR/Cas system. Nucleus March–April 2014;5(2):163–72.

Baker DA, Harkonen WS. Regulatory agency concerns in the manufacturing and testing of monoclonal antibodies for therapeutic use. Targeted Diagn Ther 1990;3:75–98.

Bao Z, HamediRad M, Xue P, Xiao H, Tasan I, Chao R, Liang J, Zhao H. Genome-scale engineering of Saccharomyces cerevisiae with single-nucleotide precision. Nat Biotechnol May 7, 2018.

Bassett AR, Tibbit C, Ponting CP, Liu JL. Highly efficient targeted mutagenesis of Drosophila with the CRISPR/Cas9 system. Cell Rep July 11, 2013;4(1):220–8.

Bishop DT. BRCA1, BRCA2, BRCA3... a myriad of breast cancer genes. Eur J Cancer 1994;30A(12):1738–9.

Bolotin A, Quinquis B, Sorokin A, Ehrlich SD. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology August 2005;151(Pt 8):2551–61.

Borel F, Kay MA, Mueller C. Recombinant AAV as a platform for translating the therapeutic potential of RNA interference. Mol Ther April 2014;22(4):692–701.

Burstein D, Harrington LB, Strutt SC, Probst AJ, Anantharaman K, Thomas BC, Doudna JA, Banfield JF. New CRISPR-Cas systems from uncultivated microbes. Nature February 9, 2017;542(7640):237–41.

Campbell IB, Macdonald SJF, Procopiou PA. Medicinal chemistry in drug discovery in big pharma: past, present and future. Drug Discov Today February 2018;23(2):219–34.

Casey G, Plummer S, Hoeltge G, Scanlon D, Fasching C, Stanbridge EJ. Functional evidence for a breast cancer growth suppressor gene on chromosome 17. Hum Mol Genet November 1993;2(11):1921–7.

CBR Pharma Insights. Monoclonal antibodies - global trends in the competitive. Technological and R&D Landscape. Research and Markets; October 2017. p. 1–57. Region: Global.

Charlesworth CT, Deshpande PS, Dever DP, Dejene B, Gomez-Ospina N, Mantri S, Pavel-Dinu M, Camarena J, Weinberg KI, Porteus MH. Identification of pre-existing adaptive immunity to Cas9 proteins in humans. BioRxiv January 5, 2018. [not peer reviewed] https://doi.org/10.1101/243345.

Chavez A, Scheiman J, Vora S, Pruitt BW, Tuttle M, Iyer PE, Lin S, Kiani S, Guzman CD, Wiegand DJ, Ter-Ovanesyan D, Braff JL, Davidsohn N, Housden BE, Perrimon N, Weiss R, Aach J, Collins JJ, Church GM. Highly efficient Cas9-mediated transcriptional programming. Nat Methods April 2015;12(4):326–8.

Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li GW, Park J, Blackburn EH, Weissman JS, Qi LS, Huang B. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. Cell December 19, 2013;155(7):1479–91.

Chen S, Sun H, Miao K, Deng C. CRISPR-Cas9: from genome editing to cancer research. Int J Biol Sci 2016;12(12):1427–36.

Cichonska A, Rousu J, Aittokallio T. Identification of drug candidates and repurposing opportunities through compound-target interaction networks. Expert Opin Drug Discov December 2015;10(12):1333–45.

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Multiplex genome engineering using CRISPR/Cas systems. Science February 15, 2013;339(6121):819–23.

Crunkhorn S. HIV: CRISPR screen identifies novel therapeutic targets. Nat Rev Drug Discov February 2, 2017;16(2):88.

DeLeo AB, Jay G, Appella E, Dubois GC, Law LW, Old LJ. Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. Proc Natl Acad Sci USA May 1979;76(5):2420–4.

Díez-Villaseñor C, Guzmán NM, Almendros C, García-Martínez J, Mojica FJ. CRISPR-spacer integration reporter plasmids reveal distinct genuine acquisition specificities among CRISPR-Cas I-E variants of Escherichia coli. RNA Biol May 2013;10(5):792–802.

DiGiusto DL, Cannon PM, Holmes MC, Li L, Rao A, Wang J, Lee G, Gregory PD, Kim KA, Hayward SB, Meyer K, Exline C, Lopez E, Henley J, Gonzalez N, Bedell V, Stan R, Zaia JA. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. Mol Ther Methods Clin Dev November 9, 2016;3:16067.

DiMasi JA, Grabowski HG, Hansen RW. Innovation in the pharmaceutical industry: new estimates of R&D costs. J Health Econ 2016;47:20–33.

Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science November 28, 2014;346(6213):1258096.

Ecker DM, Jones SD, Levine HL. The therapeutic monoclonal antibody market. MAbs 2015;7(1):9–14.

Farboud B, Meyer BJ. Dramatic enhancement of genome editing by CRISPR/Cas9 through improved guide RNA design. Genetics April 2015;199(4):959–71.

Finn JD, Smith AR, Patel MC, Shaw L, Youniss MR, van Heteren J, Dirstine T, Ciullo C, Lescarbeau R, Seitzer J, Shah RR, Shah A, Ling D, Growe J, Pink M, Rohde E, Wood KM, Salomon WE, Harrington WF, Dombrowski C, Strapps WR, Chang Y, Morrissey DV. A single administration of CRISPR/Cas9 lipid nanoparticles achieves robust and persistent in vivo genome editing. Cell Rep February 27, 2018;22(9):2227–35.

Fougerolles De AR. Delivery vehicles for small interfering RNA in vivo. Hum Gene Ther February 2008;19(2):125–32.

Friedland AE, Tzur YB, Esvelt KM, Colaiácovo MP, Church GM, Calarco JA. Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nat Methods August 2013;10(8):741–3.

Gebre M, Nomburg JL, Gewurz BE. CRISPR-Cas9 genetic analysis of virus-host interactions. Viruses January 30, 2018;10(2).

Geldhof P, Visser A, Clark D, Saunders G, Britton C, Gilleard J, Berriman M, Knox D. RNA interference in parasitic helminths: current situation, potential pitfalls and future prospects. Parasitology May 2007;134(Pt 5):609–19.

Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA, Weissman JS, Qi LS. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell July 18, 2013;154(2):442–51.

Gilbert LA, Horlbeck MA, Adamson B, Villalta JE, Chen Y, Whitehead EH, Guimaraes C, Panning B, Ploegh HL, Bassik MC, Qi LS, Kampmann M, Weissman JS. Genome-scale CRISPR-mediated control of gene repression and activation. Cell October 23, 2014;159(3):647–61.

Gratz SJ, Cummings AM, Nguyen JN, Hamm DC, Donohue LK, Harrison MM, Wildonger J, O'Connor-Giles KM. Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. Genetics August 2013;194(4):1029–35.

Gratz SJ, Wildonger J, Harrison MM, O'Connor-Giles KM. CRISPR/Cas9-mediated genome engineering and the promise of designer flies on demand. Fly (Austin) October–December, 2013;7(4):249–55.

Grissa I, Vergnaud G, Pourcel C. The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. BMC Bioinf May 23, 2007;8:172.

Guo J, Ma D, Huang R, Ming J, Ye M, Kee K, Xie Z, Na J. An inducible CRISPR-ON system for controllable gene activation in human pluripotent stem cells. Protein Cell May 2017;8(5):379–93.

Haft DH, Selengut J, Mongodin EF, Nelson KE. A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. PLoS Comput Biol November 2005;1(6).

Hale CR, Majumdar S, Elmore J, Pfister N, Compton M, Olson S, Resch AM, Glover 3rd CV, Graveley BR, Terns RM, Terns MP. Essential features and rational design of CRISPR RNAs that function with the Cas RAMP module complex to cleave RNAs. Mol Cell February 10, 2012;45(3):292–302.

Haurwitz RE, Jinek M, Wiedenheft B, Zhou K, Doudna JA. Sequence- and structure-specific RNA processing by a CRISPR endonuclease. Science September 10, 2010;329(5997):1355–8.

Henderson E. BioForce Nanosciences, Inc. Nanomedicine (Lond) June 2007;2(3):391–6.

Hille F, Richter H, Wong SP, Bratovič M, Ressel S, Charpentier E. The biology of CRISPR-cas: backward and forward. Cell March 8, 2018;172(6):1239–59.

Holt N, Wang J, Kim K, Friedman G, Wang X, Taupin V, Crooks GM, Kohn DB, Gregory PD, Holmes MC, Cannon PM. In vivo genome editing of the albumin locus as a platform for protein replacement therapy. Nat Biotechnol August 2010;28(8):839–47.

Horvath P, Barrangou R. CRISPR/Cas, the immune system of bacteria and archaea. Science January 8, 2010;327(5962):167–70.

Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell June 5, 2014;157(6):1262–78.

Hu W, Kaminski R, Yang F, Zhang Y, Cosentino L, Li F, Luo B, Alvarez-Carbonell D, Garcia-Mesa Y, Karn J, Mo X, Khalili K. RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. Proc Natl Acad Sci USA August 5, 2014;111(31):11461–6.

Hu JH, Miller SM, Geurts MH, Tang W, Chen L, Sun N, Zeina CM, Gao X, Rees HA, Lin Z, Liu DR. Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. Nature April 5, 2018;556(7699):57–63.

Hu C. Imetelstat, a telomerase inhibitor, is capable of depleting myelofibrosis hematopoietic stem cells and progenitor cells. ASH 2017.

Ishino Y, Krupovic M, Forterre P. History of CRISPR-cas from encounter with a mysterious repeated sequence to genome editing technology. J Bacteriol March 12, 2018;200(7).

Jansen R, Embden JD, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. Mol Microbiol March 2002;43(6):1565–75.

Jasin M, de Villiers J, Weber F, Schaffner W. High frequency of homologous recombination in mammalian cells between endogenous and introduced SV40 genomes. Cell December 1985;43(3 Pt 2):695–703.

Ji H, Jiang Z, Lu P, Ma L, Li C, Pan H, Fu Z, Qu X, Wang P, Deng J, Yang X, Wang J, Zhu H. Specific reactivation of latent HIV-1 by dCas9-SunTag-VP64-mediated guide RNA targeting the HIV-1 promoter. Mol Ther March 2016;24(3):508–21.

Ji P, Manupipatpong S, Xie N, Li Y. Induced pluripotent stem cells: generation strategy and epigenetic mystery behind reprogramming. Stem Cells Int 2016;2016:8415010.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science August 17, 2012;337(6096):816–21.

Jost M, Chen Y, Gilbert LA, Horlbeck MA, Krenning L, Menchon G, Rai A, Cho MY, Stern JJ, Prota AE, Kampmann M, Akhmanova A, Steinmetz MO, Tanenbaum ME, Weissman JS. Combined CRISPRi/a-–based chemical genetic screens reveal that rigosertib is a microtubule-destabilizing agent. Mol Cell October 5, 2017;68(1):210–23.

Kaminski R, Chen Y, Fischer T, Tedaldi E, Napoli A, Zhang Y, Karn J, Hu W, Khalili K. Elimination of HIV-1 genomes from human t-lymphoid cells by CRISPR/Cas9 gene editing. Sci Rep March 4, 2016;6:22555.

Kampmann M. CRISPRi and CRISPRa screens in mammalian cells for precision biology and medicine. ACS Chem Biol February 16, 2018;13(2):406–16.

Karvelis T, Gasiunas G, Siksnys V. Methods for decoding Cas9 protospacer adjacent motif (PAM) sequences: a brief overview. Methods May 15, 2017;121–122:3–8.

Kasap C, Elemento O, Kapoor TM. DrugTargetSeqR: a genomics- and CRISPR-Cas9-based method to analyze drug targets. Nat Chem Biol August 2014;10(8):626–8.

Khoshnejad M, Brenner JS, Motley W, Parhiz H, Greineder CF, Villa CH, Marcos-Contreras OA, Tsourkas A, Muzykantov VR. Molecular engineering of antibodies for site-specific covalent conjugation using CRISPR/Cas9. Sci Rep January 29, 2018;8(1):1760.

Kim DH, Rossi JJ. Strategies for silencing human disease using RNA interference. Nat Rev Genet March 2007;8(3):173–84.

Kim YB, Komor AC, Levy JM, Packer MS, Zhao KT, Liu DR. Increasing the genome-targeting scope and precision of base editing with engineered Cas9-cytidine deaminase fusions. Nat Biotechnol April 2017;35(4):371–6.

Kim SM, Yang Y, Oh SJ, Hong Y, Seo M, Jang M. Cancer-derived exosomes as a delivery platform of CRISPR/Cas9 confer cancer cell tropism-dependent targeting. J Control Release November 28, 2017;266:8–16.

Kleinstiver BP, Prew MS, Tsai SQ, Nguyen NT, Topkar VV, Zheng Z, Joung JK. Broadening the targeting range of Staphylococcus aureus CRISPR-Cas9 by modifying PAM recognition. Nat Biotechnol December 2015;33(12):1293–8.

Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JK. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. Nature January 28, 2016;529(7587):490–5.

Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature May 19, 2016;533(7603):420–4.

Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H, Nureki O, Zhang F. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature January 29, 2015;517(7536):583–8.

Koo T, Lee J, Kim JS. Measuring and reducing off-target activities of programmable nucleases including CRISPR-cas9. Mol Cells June 2015;38(6):475–81.

Kurata M, Yamamoto K, Moriarity BS, Kitagawa M, Largaespada DA. CRISPR/Cas9 library screening for drug target discovery. J Hum Genet February 2018;63(2):179–86.

La Russa MF, Qi LS. The new state of the art: cas9 for gene activation and repression. Mol Cell Biol November 2015;35(22):3800–9.

Lancrey A, Joubert A, Boulé JB. Locus specific engineering of tandem DNA repeats in the genome of Saccharomyces cerevisiaeusing CRISPR/Cas9 and overlapping oligonucleotides. Sci Rep May 8, 2018;8(1):7127.

le Sage C, Lawo S, Panicker P, Scales TME, Rahman SA, Little AS, McCarthy NJ, Moore JD. Cross BCS. Dual direction CRISPR transcriptional regulation screening uncovers gene networks driving drug resistance. Sci Rep December 18, 2017;7(1):17693.

Lee HY, Haurwitz RE, Apffel A, Zhou K, Smart B, Wenger CD, Laderman S, Bruhn L, Doudna JA. RNA-protein analysis using a conditional CRISPR nuclease. Proc Natl Acad Sci USA April 2, 2013;110(14):5416–21.

Leon LM, Mendoza SD, Bondy-Denomy J. How bacteria control the CRISPR-Cas arsenal. Curr Opin Microbiol April 2018;42:87–95.

Li J, Zhu Z. Research and development of next generation of antibody-based therapeutics. Acta Pharmacol Sin 2010;31:1198–207.

Li D, Qiu Z, Shao Y, Chen Y, Guan Y, Liu M, Li Y, Gao N, Wang L, Lu X, Zhao Y, Liu M. Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. Nat Biotechnol August 2013;31(8):681–3.

Li L, Krymskaya L, Wang J, Henley J, Rao A, Cao LF, Tran CA, Torres-Coronado M, Gardner A, Gonzalez N, Kim K, Liu PQ, Hofer U, Lopez E, Gregory PD, Liu Q, Holmes MC, Cannon PM, Zaia JA, DiGiusto DL. Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted to CCR5 control HIV-1 in vivo. Mol Ther June 2013;21(6):1259–69.

Li X, Wang Y, Liu Y, Yang B, Wang X, Wei J, Lu Z, Zhang Y, Wu J, Huang X, Yang L, Chen J. Base editing with a Cpf1-cytidine deaminase fusion. Nat Biotechnol April 2018;36(4):324–7.

Lin Y, Wu J, Gu W, Huang Y, Tong Z, Huang L, Tan J. Exosome-liposome hybrid nanoparticles deliver CRISPR/Cas9 system in MSCs. Adv Sci (Weinh) January 30, 2018;5(4):1700611.

Liu X, Zhao Y. CRISPR/Cas9 genome editing: Fueling the revolution in cancer immunotherapy. Curr Res Transl Med May 2018;66(2):39–42.

Liu X, Zhang Y, Cheng C, Cheng AW, Zhang X, Li N, Xia C, Wei X, Liu X, Wang H. CRISPR-Cas9-mediated multiplex gene editing in CAR-T cells. Cell Res January 2017;27(1):154–7.

Liu T, Pan S, Li Y, Peng N, She Q. Type III CRISPR-cas system: introduction and its application for genetic manipulations. Curr Issues Mol Biol 2018;26:1–14.

Liu JKH. The history of monoclonal antibody development - progress, remaining challenges and future innovations. Ann Med Surg (Lond) December 2014;3(4):113–6.

Ma W. Telomerase inhibition impairs self-renewal of b-Catenin activated myeloproliferative neoplasm progenitors. ASH 2017.

Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH, Joung JK. CRISPR RNA-guided activation of endogenous human genes. Nat Methods October 2013;10(10):977–9.

Maggon K. Monoclonal antibody "Gold Rush". Curr Med Chem 2007;14(8):1978–87.

Makarova KS, Aravind L, Wolf YI, Koonin EV. Unification of Cas protein families and a simple scenario for the origin and evolution of CRISPR-Cas systems. Biol Direct July 14, 2011;6:38.

Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, Moineau S, Mojica FJ, Wolf YI, Yakunin AF, van der Oost J, Koonin EV. Evolution and classification of the CRISPR-Cas systems. Nat Rev Microbiol June 2011;9(6):467–77.

Marraffini LA, Sontheimer EJ. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. Nat Rev Genet March 2010;11(3):181–90.
Marraffini LA, Sontheimer EJ. Self versus non-self discrimination during CRISPR RNA-directed immunity. Nature January 28, 2010;463(7280):568–71.
Mojica FJ, Díez-Villaseñor C, García-Martínez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J Mol Evol February 2005;60(2):174–82.
Moore JK, Haber JE. Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in Saccharomyces cerevisiae. Mol Cell Biol May 1996;16(5):2164–73.
Mullen E. An $850,000 price tag on gene therapy Shouldn't Freak You out—yet. MIT Technol Review January 4, 2018.
Nam KH, Huang Q, Ke A. Nucleic acid binding surface and dimer interface revealed by CRISPR-associated CasB protein structures. FEBS Lett November 16, 2012;586(22):3956–61.
Nam KH, Haitjema C, Liu X, Ding F, Wang H, DeLisa MP, Ke A. Cas5d protein processes pre-crRNA and assembles into a cascade-like interference complex in subtype I-C/Dvulg CRISPR-Cas system. Structure September 5, 2012;20(9):1574–84.
Nelson AL, Dhimolea E, Reichert JM. Development trends for human monoclonal antibody therapeutics. Nat Rev Drug Discov 2010;9(10):767–74.
Niewoehner O, Jinek M. Specialized weaponry: how a type III-A CRISPR-cas system excels at combating phages. Cell Host Microbe September 13, 2017;22(3):258–9.
No Authors Listed. CRISPR meets CAR t-cell therapy. Cancer Discov May 2017;7(5).
Park RJ, Wang T, Koundakjian D, Hultquist JF, Lamothe-Molina P, Monel B, Schumann K, Yu H, Krupzcak KM, Garcia-Beltran W, Piechocka-Trocha A, Krogan NJ, Marson A, Sabatini DM, Lander ES, Hacohen N, Walker BD. A genome-wide CRISPR screen identifies a restricted set of HIV host dependency factors. Nat Genet February 2017;49(2):193–203.
Pattanayak V, Guilinger JP, Liu DR. Determining the specificities of TALENs, Cas9, and other genome-editing enzymes. Methods Enzymol 2014;546:47–78.
Perez-Pinera P, Ousterout DG, Brunger JM, Farin AM, Glass KA, Guilak F, Crawford GE, Hartemink AJ, Gersbach CA. Synergistic and tunable human gene activation by combinations of synthetic transcriptionfactors. Nat Methods March 2013;10(3):239–42.
Petricciani JC. An overview of safety and regulatory aspects of the new biotechnology. Regul Toxicol Pharmacol December 1983;3(4):428–33.
Przybilski R, Richter C, Gristwood T, Clulow JS, Vercoe RB, Fineran PC. Csy4 is responsible for CRISPR RNA processing in Pectobacterium atrosepticum. RNA Biol May–June 2011;8(3):517–28.
Qi L, Haurwitz RE, Shao W, Doudna JA, Arkin AP. RNA processing enables predictable programming of gene expression. Nat Biotechnol October 2012;30(10):1002–6.
Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nat Protoc November 2013;8(11):2281–308.
Resnick MA. The repair of double-strand breaks in DNA; a model involving recombination. J Theor Biol June 1976;59(1):97–106.
Richter C, Chang JT, Fineran PC. Function and regulation of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) systems. Viruses October 19, 2012;4(10):2291–311.
Richter H, Lange SJ, Backofen R, Randau L. Comparative analysis ofCas6b processing and CRISPR RNA stability. RNA Biol May 2013;10(5):700–7.
Richter H, Randau L, Plagens A. Exploiting CRISPR/Cas: interference mechanisms and applications. Int J Mol Sci July 12, 2013;14(7):14518–31.
Rodrigues NR, Rowan A, Smith ME, Kerr IB, Bodmer WF, Gannon JV, Lane DP. p53 mutations in colorectal cancer. Proc Natl Acad Sci USA October 1990;87(19):7555–9.
Roth DB, Wilson JH. Nonhomologous recombination in mammalian cells: role for short sequence homologies in the joining reaction. Mol Cell Biol December 1986;6(12):4295–304.
Sarisozen C, Salzano G, Torchilin VP. Lipid-based siRNA delivery systems: challenges, promises and solutions along the long journey. Curr Pharm Biotechnol 2016;17(8):728–40.
Senís E, Fatouros C, Große S, Wiedtke E, Niopek D, Mueller AK, Börner K, Grimm D. CRISPR/Cas9-mediated genome engineering: an adeno-associated viral (AAV) vector toolbox. Biotechnol J November 2014;9(11):1402–12.

Shah SA, Hansen NR, Garrett RA. Distribution of CRISPR spacer matches in viruses and plasmids of crenarchaeal acidothermophiles and implications for their inhibitory mechanism. Biochem Soc Trans February 2009;37(Pt 1):23–8.
Shajari N, Mansoori B, Davudian S, Mohammadi A, Baradaran B. Overcoming the challenges of siRNA delivery: nanoparticle strategies. Curr Drug Deliv 2017;14(1):36–46.
Sharma R, Anguela XM, Doyon Y, Wechsler Thomas, DeKelver RC, Sproul S, Paschon DE, Miller JC, Davidson RJ, Shivak D, Zhou S, Rieders J, Gregory PD, Holmes MC, Rebar EJ, High KA. In vivo genome editing of the albumin locus as a platform for protein replacement therapy. Blood October 8, 2015;126(15).
Shegokar R, Al Shaal L, Mishra PR. SiRNA delivery: challenges and role of carrier systems. Pharmazie May 2011;66(5):313–8.
Sherkow JS. The CRISPR patent landscape: past, present, and future. CRISPR J 2018;1(1):5–9.
Shi J, Wang E, Milazzo JP, Wang Z, Kinney JB, Vakoc CR. Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains. Nat Biotechnol June 2015;33(6):661–7.
Sorek R, Kunin V, Hugenholtz P. CRISPR–a widespread system that provides acquired resistance against phages in bacteria and archaea. Nat Rev Microbiol March 2008;6(3):181–6.
Stern A, Mick E, Tirosh I, Sagy O, Sorek R. CRISPR targeting reveals a reservoir of common phages associated with the human gut microbiome. Genome Res October 2012;22(10):1985–94.
Sullman SF. Regulatory issues with respect to monoclonal antibodies–an industry perspective. Dev Biol Stand 1990;71:207–11.
Sun W, Ji W, Hall JM, Hu Q, Wang C, Beisel CL, Gu Z. Self-assembled DNA nanoclews for the efficient delivery of CRISPR-Cas9 for genome editing. Angew Chem Int Ed Engl October 5, 2015;54(41):12029–33.
Szostak JW, Orr-Weaver TL, Rothstein RJ, Stahl FW. The double-strand-break repair model for recombination. Cell May 1983;33(1):25–35.
Tebas P, Stein D, Tang WW, Frank I, Wang SQ, Lee G, Spratt SK, Surosky RT, Giedlin MA, Nichol G, Holmes MC, Gregory PD, Ando DG, Kalos M, Collman RG, Binder-Scholl G, Plesa G, Hwang WT, Levine BL, June CH. Genomic editing of the HIV-1 coreceptor CCR5 in adult hematopoietic stem and progenitor cells using zinc finger nucleases. N Engl J Med March 6, 2014;370(10):901–10.
Thorpe R. Monoclonal antibodies: clinical and regulatory issues. Trends Biotechnol February 1993;11(2):40–2.
Tiemann K, Rossi JJ. RNAi-based therapeutics-current status, challenges and prospects. EMBO Mol Med June 2009;1(3):142–51.
Tsai SQ, Nguyen NT, Malagon-Lopez J, Topkar VV, Aryee MJ, Joung JK. CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR-Cas9 nuclease off-targets. Nat Methods June 2017;14(6):607–14.
Tsui CK, Gupta A, Bassik MC. Finding host targets for HIV therapy. Nat Genet January 31, 2017;49(2):175–6.
Ul Ain Q, Chung JY, Kim YH. Current and future delivery systems for engineered nucleases: ZFN, TALEN and RGEN. J Control Release May 10, 2015;205:120–7.
Uppada V, Gokara M, Rasineni GK. Diagnosis and therapy with CRISPR advanced CRISPR based tools for point of care diagnostics and early therapies. Gene May 20, 2018;656:22–9.
Uprichard SL. The therapeutic potential of RNA interference. FEBS Lett October 31, 2005;579(26):5996–6007.
Walchli S, Sioud M. Vector-based delivery of siRNAs: in vitro and in vivo challenges. Front Biosci May 1, 2008;13:3488–93.
Wang J, Quake SR. RNA-guided endonuclease provides a therapeutic strategy to cure latent herpesviridae infection. Proc Natl Acad Sci USA September 9, 2014;111(36):13157–62.
Wang QZ, Lv YH, Diao Y, Xu R. The design of vectors for RNAi delivery system. Curr Pharm Des 2008;14(13):1327–40.
Wang W, Ye C, Liu J, Zhang D, Kimata JT, Zhou P. CCR5 gene disruption via lentiviral vectors expressing Cas9 and single guided RNA renders cells resistant to HIV-1 infection. PLoS One December 26, 2014;9(12).
Wang Z, Pan Q, Gendron P, Zhu W, Guo F, Cen S, Wainberg MA, Liang C. CRISPR/Cas9-Derived mutations both inhibit HIV-1 replication and accelerate viral escape. Cell Rep April 19, 2016;15(3):481–9.

Wang LW, Trudeau SJ, Wang C, Gerdt C, Jiang S, Zhao B, Gewurz BE. Modulating gene expression in Epstein-Barr virus (EBV)-Positive B cell lines with CRISPRa and CRISPRi. Curr Protoc Mol Biol January 16, 2018;121:31.13.1–8.

White MK, Hu W, Khalili K. Gene editing approaches against viral infections and strategy to prevent occurrence of viral escape. PLoS Pathog December 8, 2016;12(12).

Wiedenheft B, Zhou K, Jinek M, Coyle SM, Ma W, Doudna JA. Structural basis for DNase activity of a conserved protein implicated in CRISPR-mediated genome defense. Structure June 10, 2009;17(6):904–12.

Wright AV, Nuñez JK, Doudna JA. Biology and applications of CRISPR systems: harnessing nature's toolbox for genome engineering. Cell January 14, 2016;164(1–2):29–44.

Wright AV, Liu JJ, Knott GJ, Doxzen KW, Nogales E, Doudna JA. Structures of the CRISPR genome integration complex. Science September 15, 2017;357(6356):1113–8.

Wu Y, Zhou H, Fan X, Zhang Y, Zhang M, Wang Y, Xie Z, Bai M, Yin Q, Liang D, Tang W, Liao J, Zhou C, Liu W, Zhu P, Guo H, Pan H, Wu C, Shi H, Wu L, Tang F, Li J. Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. Cell Res January 2015;25(1):67–79.

Yang X, Boehm JS, Yang X, Salehi-Ashtiani K, Hao T, Shen Y, Lubonja R, Thomas SR, Alkan O, Bhimdi T, Green TM, Johannessen CM, Silver SJ, Nguyen C, Murray RR, Hieronymus H, Balcha D, Fan C, Lin C, Ghamsari L, Vidal M, Hahn WC, Hill DE, Root DE. A public genome-scale lentiviral expression library of human ORFs. Nat Methods June 26, 2011;8(8):659–61.

Yin C, Zhang T, Qu X, Zhang Y, Putatunda R, Xiao X, Li F, Xiao W, Zhao H, Dai S, Qin X, Mo X, Young WB, Khalili K, Hu W. In vivo excision of HIV-1 provirus by saCas9 and multiplex single-guide RNAs in animal models. Mol Ther May 3, 2017;25(5):1168–86.

Zhang Y, Sastre D, Wang F. CRISPR/Cas9 genome editing: a promising tool for therapeutic applications of induced pluripotent stem cells. Curr Stem Cell Res Ther 2018;13(4):243–51.

Zhao N, Wang G, Das AT, Berkhout B. Combinatorial CRISPR-Cas9 and RNA interference attack on HIV-1 DNA and RNA can lead to cross-resistance. Antimicrob Agents Chemother November 22, 2017;61(12).