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Advances in CRISPR-Cas based genome engineering

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Development of the CRISPR-Cas systems has catalyzed rapid advancements in the field of genome engineering. Recently, the technology has seen various new developments, including the successful use in RNA-targeting, the development of more precise genome editing reagents, and breakthroughs in applications to gene therapy and regenerative medicine. These rapid developments, when coupled with its ease of use and high efficiency, are spurring widespread adoption of the technique, and in fact, use of CRISPR-Cas technology towards gene therapy clinical trials is already underway. In this review, we provide an overview of the CRISPR-Cas systems for genome engineering, and in particular, stress key toolsets and methodologies that we believe will have particularly broad impact on both basic science and translational research.

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Introduction

Clustered regularly interspaced short palindromic repeats – CRISPR associated protein (CRISPR-Cas) systems are naturally occurring bacterial adaptive immune systems designed to combat invading viruses and nucleic acids [1–3]. Some CRISPR-Cas systems utilize a single effector Cas protein in complex with CRISPR RNAs (crRNAs) to scan and cleave invading nucleic-acid sequences. For instance, the Type II system from *Streptococcus pyogenes* (SpCas9), recognizes invading dsDNA sequences through both base-pairing with the CRISPR RNA (crRNAs), and the presence of protospacer adjacent motifs (PAM) immediately downstream of the target region. In these systems, pre-crRNAs are processed with the help of a trans-activating crRNA (tracrRNA), leading to the formation of a tracrRNA:crRNA:Cas9 complex that creates double stranded breaks at the target loci [4]. In genome-engineering applications, this tracrRNA-crRNA complex has been replaced by a chimeric single guide RNA (sgRNA), which consists of constant and variable regions – here the constant portion facilitates binding of the Cas9 and the variable spacer portion guides target sequence recognition via simple Watson-Crick base pairing [4]. In principle, sgRNAs can be designed to target a sequence of about 20 nucleotides, present immediately upstream of the PAM. It is important to note though, that the activity of the Cas9-sgRNA complex varies with the specific sgRNA used. Taken together, the ease by which Cas9 or Cas9-like single-effector proteins can be programmed to target a desired locus by expressing the corresponding sgRNA, and its applicability in editing human cells [5–8] have made it a versatile and powerful tool for genome engineering.

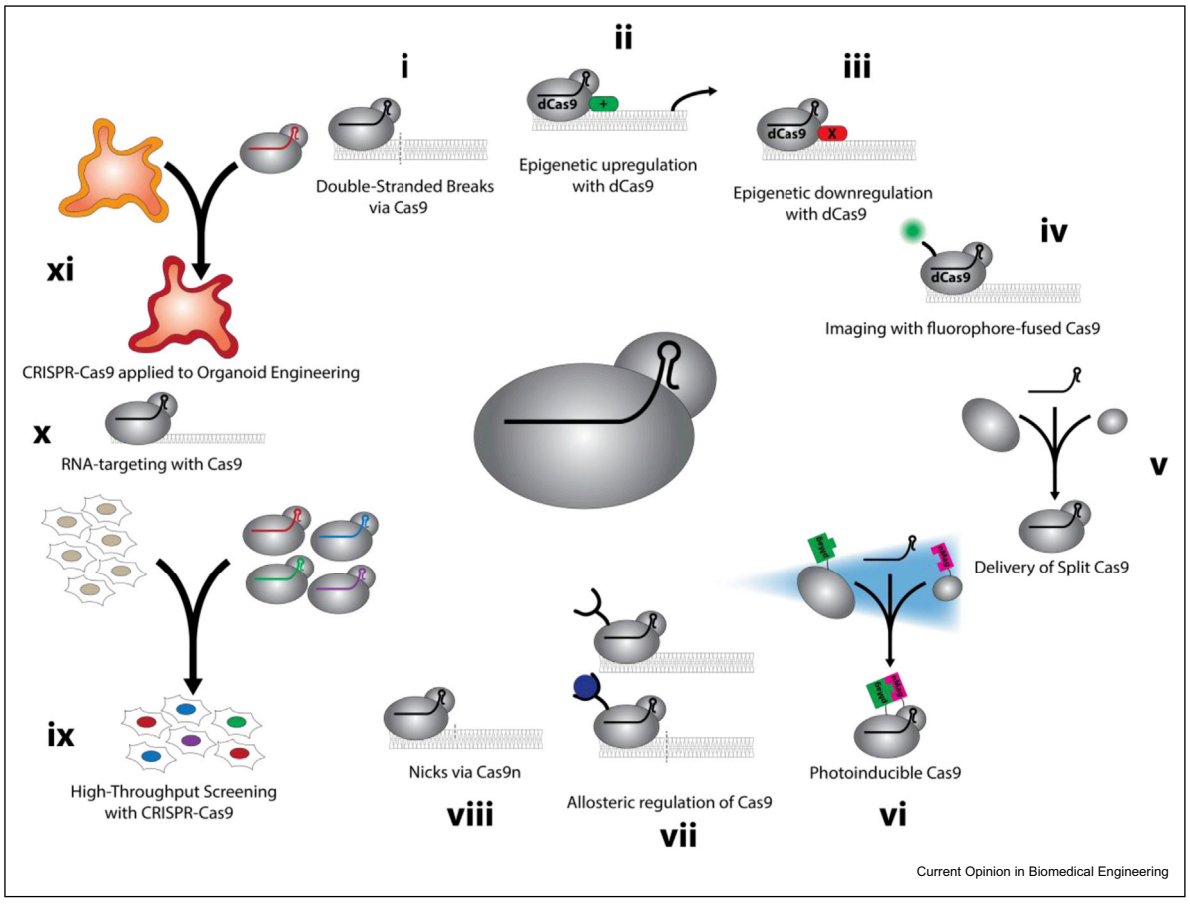
In practice, genome editing via CRISPR-Cas9 makes use of endogenous cellular repair mechanisms that respond to double stranded breaks (DSB). Co-delivery of the Cas9 nuclease, in plasmid or mRNA form, with a corresponding sgRNA will generate DSBs at a locus of interest, and the pathway subsequently used to repair this break – Non-Homologous End Joining (NHEJ) or Homology Directed Repair (HDR) - depends on the cell cycle phase and the presence or absence of a homologous sequence. NHEJ is an error prone process that creates insertions and deletions (indels) while HDR can be used to insert precise genetic modifications via the addition of a synthetic donor. This fundamental system has been thoroughly explored, and has been used to perform targeted single and multiplexed gene deletions and recombinations in various *in vitro* and *in vivo* models [5,6,9]. Notably, this system does have several limitations, including the generation of off-target DSBs, and limitation to loci expressing the PAM sequence. However, significant progress has been made in addressing these shortcomings. In the following sections we provide a summary of many of these recently-engineered forms of the CRISPR-Cas systems, as well as their existing and potential applications (Figure 1), with several particularly notable advances highlighted in greater detail.

CRISPR-Cas toolset

Genome engineering

From a clinical perspective, increasing the efficiency of gene editing will be vital. To achieve targeted gene editing while limiting off target effects, researchers have explored multiple approaches. One strategy is to regulate Cas9 activity both spatially and temporally. Spatial regulation can be achieved by either delivering the Cas9

Figure 1



CRISPR-Cas9 genome and transcriptome engineering toolset. i) Cas9 can be used to generate double-stranded breaks in DNA, which can subsequently be repaired by HDR or NHEJ mechanisms, allowing for site-specific genome editing [4]. ii) Fusion of nuclease-null dCas9 to a transcription activator can upregulate gene expression without requiring genome-modification [24]. iii) Fusion of dCas9 to a transcription repressor can downregulate gene expression without requiring genome-modification [24]. iv) Fusion of fluorophores to dCas9 can allow for real-time visualization of DNA and RNA dynamics [32]. v) Delivery of Cas9 or modified Cas9 can be facilitated by splitting the two nuclease lobes, and delivering them independently using viral delivery systems [12,37,38]. vi) Fusion of spCas9 with light-responsive dimerization proteins allows for assembly of components in response to blue light, generating photoinducible Cas9 [14–16]. vii) Cas9 can be modified to incorporate allosteric domains, allowing Cas9 activity to be controlled through the addition or removal of specific small-molecules [35]. viii) Mutation of the catalytically active Cas9 RuvC or HNH domains produces Cas9n, which is able to generate single-stranded nicks rather than a double-stranded break [4]. ix) gRNA libraries can be used for enabling high-throughput genetic screens [41]. x) Targeting of RNA with Cas9 can be achieved by co-delivering modified PAM sequences (PAMmers) to promote ssRNA binding [29]. xi) By using CRISPR-Cas9 to insert or knockout specific genes in organoids, many human tissue-specific disease models can be developed.

to a specific tissue, or by placing it downstream of a tissue-specific promoter. In contrast, temporal regulation can been achieved by making use of the Tet-on system, in which the expression of Cas9 is placed under a promoter with a Tetracycline Response Element (TRE), such that Cas9 expression is induced with doxycycline [10]. This system, dubbed inducible CRISPR (iCRISPR), has been applied to human pluripotent stem cells to generate hPSC knockouts at different stages of differentiation, making it possible to carry out systematic genetic studies in hPSCs [10]. *In vivo* iCRISPR application has also shown promising results, with doxycycline-induced Cas9 expression producing robust gene disruption in multiple tissues, and inducible Cas9 nickase (Cas9n) modulating the frequency and size of target gene modifications [11]. In addition to iCRISPR, alternative systems have also been developed in which Cas9 has been engineered to respond to other small molecules such as Rapamycin [12] and 4-hydroxytamoxifen (4-HT) [13]. Moreover, researchers have also engineered light responsive Cas9 systems, with one approach using a fusion of the light-inducible heterodimerizing proteins CRY2 and CIB1 [14], and the other using a photoinducible dimerization system called Magnets [15,16].

In addition, several groups have also worked towards improving Cas9 precision. Studies of SpCas9 have

revealed that it exhibits high specificity in the first 8–12 residues upstream of the PAM, but tolerates mismatches in the remaining residues. Reductions in off-target mutagenesis have been achieved both by varying the dosage of SpCas9 and sgRNA, and through the use of truncated guide RNAs [17]. Additionally, mutating the catalytic residues in either of the Cas9 endonuclease domains (RuvC: D10A; HNH: H840A) leads to the formation of nickases (Cas9n) that create single stranded DNA breaks [4]. The pairing of Cas9n with two gRNAs, to create two single stranded breaks on opposite strands, can be used to reduce off target effects [18,19]. Analogously, high specificity is also achieved through the fusion of the FokI nuclease to dCas9 to produce a ZFN/TALEN-like programmable nuclease that is catalytically active only when two dCas9-FokI complexes come within 15–25 nucleotides of each other [20]. More recently, rational engineering of the Cas9 has produced a variant that stabilizes single stranded DNA and exhibits reduced tolerance for gRNA-DNA mismatches [21,22]. Furthermore, the addition of a programmable DNA binding domain (pDBD) to a mutant Cas9 with attenuated DNA binding affinity has produced a modular, chimeric nuclease with a broader sequence-targeting range and improved precision that can be used to make edits at nearly any genomic locus [23].

Epigenome engineering

In nature, the primary role of the Cas9 is to cleave foreign DNA sequences, but engineered forms have been generated to specifically exploit its RNA-guided DNA-targeting ability. For instance, inactivation of catalytic domains results in a protein, dCas9, which lacks nuclease activity. Subsequent fusion with other effector domains has led to the development of important tools that can be used in applications such as regulating transcription and creating epigenetic modifications, among others. By fusing the dCas9 with the Kruppel-associated box (KRAB) domain [24], high levels of transcriptional repression can be achieved, while fusion with four copies of the transcription activator VP16 (VP64) or a single p65 activation domain (p65AD) can enable target gene activation [24]. Simultaneous activation and repression of genes without altering chromosome structure has also become possible with the use of engineered gRNA scaffolds. In addition, fusing the catalytically active domains of acetyltransferase (p300) and DNA methyltransferase (DNMT3A) to the dCas9 has made it possible to achieve targeted chromatin modulation by acetylation or methylation [25,26]. Moreover, by combining riboswitches with the gRNA and making use of the dCas9 and dCas9-VP64 variants, researchers have engineered 'signal conductors' and 'logic gates' that can regulate the transcription of endogenous genes in response to external or internal signals. These in turn have been used to enable rewiring of cellular signaling networks by creating synthetic links between otherwise non-interacting cellular pathways [27].

Transcriptome engineering

The CRISPR-Cas9 system targets dsDNA, with the PAM sequence being responsible for both recruiting the Cas9-gRNA complex and activating the nuclease domain [28]. To target ssRNA, it was found to be correspondingly necessary to engineer PAMmers (PAM presenting oligonucleotides) that bound to complementary ssRNA. Using this system, researchers were able to achieve mRNA cleavage using Cas9. Furthermore, designing PAMmers against regions of ssRNA with corresponding genomic DNA that did not contain the PAM allowed for Cas9 to selectively recognize and target ssRNA. Though this leads to a mismatched 5′-NGG-3′ (of the PAMmer) during the formation of the PAMmer-ssRNA hybrid, ssRNA-specific targeting is achieved [29]. Utilizing this approach, researchers have recently modified the dCas9 to create a nuclear localized RNA-targeting Cas9 (RCas9) that can be used for live cell tracking of mRNA in a programmable manner [30]. The RCas9 binds to target mRNA in the nucleus with the help of the sgRNA and the PAMmer, and is then exported with the mRNA. During this process, conjugation of a fluorophore with the RCas9 makes it possible to visualize the mRNA localization dynamics in a living cell without the need for genetically encoded tags.

Identification of other naturally occurring CRISPR-Cas systems has also helped significantly in furthering the development of RNA targeting systems. For instance, a CRISPR-Cas9 effector, C2c2, from the bacterium *Leptotrichia shahii*, was characterized [31] and programmed to degrade ssRNA via a 28 nucleotide guide sequence. Additionally, mutation of its catalytically active residues led to the creation of the dC2c2, which, like the dCas9, could have a range of applications.

Cas9-based RNA targeting is still relatively unexplored and will have vast potential. Although RNA targeting has been achieved using siRNA, use of CRISPR-Cas systems can feasibly provide greater specificity and functionality. In the future, design of novel Cas9 or C2c2 effectors could not only modulate various RNA processing steps but also allow for spatial regulation of RNA targeting. Furthermore, precise RNA targeting might be a safer option for gene therapy when compared to DNA targeting, as it provides transient modulation of gene expression, and avoids the risk of generating permanent off-target mutations.

Genome imaging

Notably, dCas9 has also been tailored for genome imaging applications, as fusion of the dCas9 with

fluorophores allows for visualization of chromosome packaging in live cells. For instance, by using an EGFP-dCas9 construct, researchers have successfully observed chromosome dynamics such as telomere elongation and disruption, as well as the sub-nuclear localization of genomic loci [32]. Moreover, by binding engineered sgRNA scaffolds of dCas9 to fluorescent proteins of various colors, the recently developed CRISPRainbow system enables 3D fluorescent labeling of genomic structure in living cells [33]. Additionally, the fusion of a FRET pair of fluorophores to the Cas9 and target DNA strand allows for observation of real-time Cas9-RNA and DNA interaction dynamics, and has provided novel insights into target recognition and rejection kinetics and the effect of mismatches on these rates [34].

Cas9 engineering

Another important avenue by which Cas9 utility has been expanded is via Cas9 protein engineering. For instance, hotspots that tolerate insertions have been identified in the Cas9 [35], and combinatorial insertions of orthogonal domains at these locations have enabled new programmability. Furthermore, by mutating residues in the PAM binding domain of the spCas9, researchers have generated Cas9 variants with altered PAM sequences that are able to target several sites that remain elusive to the wt SpCas9 [22,36]. In addition, split Cas9s have also been engineered by separating the α-helical lobe and the nuclease lobe of the SpCas9. This architecture makes it possible, for example, to deliver the two components of the SpCas9 in viral-vector systems such as AAVs with limited packaging capacity, such that they are transported separately, but assemble *in vivo* into a functional Cas9 [12,37,38].

Having provided a summary of the core CRISPR-Cas toolset, we will now discuss in greater detail a selection of methodologies and applications enabled by these which we believe to have particularly broad potential in basic science and therapeutic applications.

CRISPR-Cas applications

High-throughput functional genomics

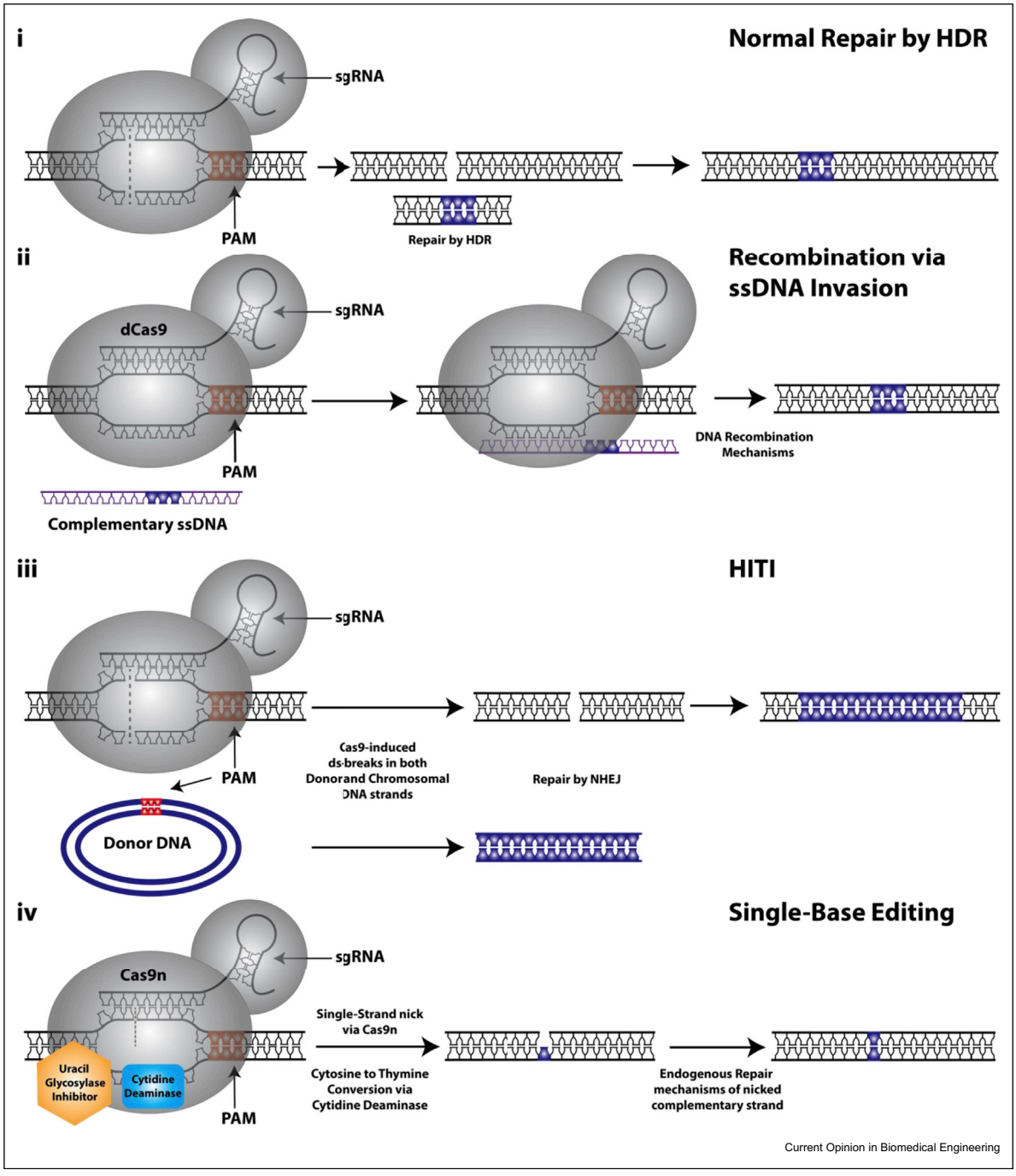
Genetic screens have traditionally been conducted using DNA mutagens and RNAi, and although these techniques have aided in characterizing many prominent biological pathways, they come with several limitations. For instance, use of DNA mutagens is imprecise, as it is extremely difficult to determine the exact location of a mutation that causes a particular phenotype, and while RNAi inhibition is more targeted, knockdown tends to be incomplete, showing variability between experiments and providing only temporary inhibition of gene expression. In contrast, the CRISPR-Cas9 system combines the permanent nature of DNA mutagens with the programmability of RNAi. Specifically, unlike RNAi [39,40], CRISPR-Cas9 can be used to either completely knockdown a gene or modulate its expression via fusion of the dCas9 with an appropriate effector.

Applicability of CRISPR-Cas9 is expanded further when considering that advances in DNA sequencing techniques and array based synthesis technologies have enabled generation of large pools of gRNA libraries that, when used to conduct genetic screens, allow for the expression and phenotypic analysis of thousands of genes simultaneously. For instance, genome scale CRISPR-Cas9 libraries have enabled high-throughput screening of cancer cells to identify genes that confer drug resistance [41]. Additionally, researchers have characterized the role of p53 and ERα-bound enhancers in their native context by making use of a gRNA pool to target their respective binding sites in enhancer regions [42], thereby expanding the applicability of Cas9 to non-coding genome sequences. Using high-throughput CRISPR screens, researchers have also identified human genes that are putatively essential for cellular function and provide insight into the genetic susceptibilities of the cell [43]. In the near future, combinatorial screens targeting two or more genes along with non-coding sequences could be used to uncover genetic interactions and provide a robust understanding of cellular pathways. Furthermore, multiplexed activation and repression of genes belonging to different pathways could help mimic disease phenotypes and prove to be useful for drug screening applications. However, some issues still need to be addressed, including estimation of off-target rates of the computationally designed sgRNAs as well as interpretation of data obtained from the genetic screens by the design of sensitive readout methods and improved tools for statistical analysis.

Precision genome engineering and HDR-independent editing

Precise, site-specific genome editing is essential for successfully correcting mutations in genetic disorders. While the CRISPR-Cas system can be used to create a precise double stranded break (DSB) at a given locus, subsequent repair may occur via either the desired HDR or the indel-forming NHEJ mechanisms. As introducing double-stranded breaks stimulates the frequency of HDR events [44], their creation using a Cas9 has become an essential first step to improving HDR frequency (Figure 2a). However, the efficiency of HDR is typically low. Point mutations are corrected with about 0.1−5% efficiency under therapeutically relevant conditions, and homologous recombination machinery is typically diminished in activity in post-mitotic cells. From a clinical perspective, predicting the exact outcome of genome editing is essential, making it important to either work towards shifting the balance from NHEJ to HDR, or to develop alternative approaches to correct point mutations.

Figure 2



Precision genome engineering via CRISPR-Cas9 i) HDR mediated repair: A ds-break is induced by Cas9, stimulating endogenous HDR mechanisms to enable precise repair. ii) Enhancement of DNA recombination with ss-donor DNA: A strand of ssDNA complementary to the non-target strand is delivered with dCas9. The PAM-distal non-target strand anneals to the ssDNA, stimulating recombination mechanisms without inducing DNA breaks [46]. iii) homology independent target integration (HITI): Donor DNA containing a PAM sequence identical to the location of interest is delivered, and Cas9 induces ds-breaks in both the chromosomal and donor DNA. Endogenous NHEJ mechanisms then join the strands during repair [47]. iv) Base-editing using a modified Cas9n: Cas9n is fused with cytidine deaminase and uracil glycosylase inhibitor. After being directed to a specific location, conversion of cytosine to thymine is performed by the cytidine deaminase, while the uracil glycosylase inhibitor prevents reversion by natural cellular repair systems. In parallel, Cas9n nicks the strand complementary to the sgRNA, stimulating complementary conversion by endogenous repair mechanisms [48].

With regards to improving HDR rates, researchers recently achieved a multifold increase by suppressing key molecules involved in NHEJ (KU70, KU80 and DNA ligase IV) [45]. Additionally, studying the dynamics of Cas9-gRNA-DNA interaction has yielded information on the asymmetric dissociation of Cas9 from DNA after cleavage. Using this information, researchers have developed ssDNA donors complementary to the non-target strand, which is released first from the Cas9, and delivery of these ssDNA donors with the Cas9 resulted in an increase of HDR rates to 60% [46].

On the front of non-HDR based methods, use of dCas9 with the aforementioned ssDNA system led to recombination rates of up to 0.7% without the introduction of strand breaks (Figure 2b). Researchers have also demonstrated the use of the homology independent targeted integration (HITI) strategy for robust DNA knock-in in both mitotic and post mitotic cells (Figure 2c). HITI is NHEJ-based and relies on the ability of a blunt ended donor to insert itself at the site of a double stranded break. After encouraging results in proof-of-concept experiments using cultured primary neurons, this technique was applied to a rat model of retinitis pigmentosa to restore function of the Mertk gene, responsible for the regulation of phagocytosis of rod outer segment membranes in the retinal pigment epithelium. Restoration of function was noticed only in the HITI donor and not in the case of the HDR donor, highlighting applicability towards editing post-mitotic cells [47].

Very recently, researchers have also developed 'base editors' that can mediate the conversion of Cytosine to Thymine on a single nucleic acid strand by fusing the dCas9 to a cytidine deaminase enzyme. This system encounters interference from natural cellular repair responses, and to resolve this, a more advanced version was created by fusing the above system to a uracil glycosylase inhibitor. Subsequently, to stimulate editing of the complementary strand, the system was generated by fusion to a Cas9 nickase (Cas9n) rather than dCas9 (Figure 2d), enabling site specific manipulation of DNA with an efficiency of 15–75% while minimizing indel formation [48]. Thus, by efficient and selective targeting of disease-relevant mutations in human cells, base editing has emerged as a powerful tool that has expanded the scope of genome engineering.

Therapeutic genome engineering

Several CRISPR-Cas9 based proof of concept studies have demonstrated successful editing of disease causing genes in disorders such as Duchenne Muscular Dystrophy [49–52], Sickle Cell Anemia [53] and B-thalassemia [54–57], both *in vivo* and *ex vivo*. In addition to the treatment of genetic disorders, several preventive therapies could be developed following proof of concept studies such as CCR5 knockouts in T-cells and induced pluripotent stem cells (iPSCs) conferring HIV resistance [7,58,59]. Below we further highlight cancer immunotherapy, an area where CRISPR-Cas9 technology is already having an impact in human clinical translation.

Cancer immunotherapy uses the components of the immune system to combat cancers, usually by enhancing the body's own immune response against cancerous cells using either antibodies or engineered T-cells. Typically, T-cell based therapy involves extraction of the immune cells from a patient followed by re-infusion after enrichment, editing or treatment. The first clinical trial involving the CRISPR-Cas9 system began in October 2016, aiming to treat a patient suffering from an aggressive form of lung cancer [60]. After collecting peripheral lymphocytes from the patient, the gene coding for Programmed Cell Death Protein 1 (PD-1) was knocked out using CRISPR-Cas9, and cells were selected, expanded *in vitro* and infused back into the patient. Since PD-1 plays an important role in halting the T-cell immune response, knocking it out would theoretically improve the ability of the T-cells to eliminate cancer cells. Similar PD-1 knockout T-cell based therapies have been approved for the treatment of bladder cancer, renal cell carcinoma and prostrate cancer [61]. In addition, a similar clinical trial was recently approved by the Recombinant DNA Advisory Committee (RAC) at the U.S. National Institutes of Health, aiming to target cancers by making multiple edits in autologous T cells [62]. The trial will recruit patients suffering from myeloma, sarcoma, and melanoma, and like its predecessor, will collect, genome-edit, and re-infuse patient-derived T-cells. Specifically, a gene coding for the NY-ESO-1 receptor will be inserted, enabling targeting of the tumor-specific NY-ESO-1 protein. Because such engineered T-cells have been shown to exhibit reduced effectiveness over time, the CRISPR-Cas9 system will also be used to knock out PD-1 along with two other genes that encode portions of the T-cell primary receptor to improve performance. Though promising, important aspects will need to be monitored during these trials such as the immune reaction to the Cas9 enzyme, non-specific genomic edits, and possible translocations when involving multiple edits.

Organoid engineering

Attempts to grow functional tissues and organoids have been ongoing for decades, and have culminated in recent breakthroughs in which *in vitro* stem cell cultures were found to self-organize into 3D organoid structures with many vivo tissue characteristics [63]. Since then, multiple organoid systems have been developed and explored, including models of the intestines, brain, kidney, liver, and retina [64]. With the advances in

CRISPR-Cas technology, many organoid systems have been combined with genome engineering techniques to produce increasingly sophisticated disease models. For instance, selective gene deletion in an organoid model of a mouse medullary-collecting duct revealed that the *Akap11* gene, which encodes for the AKAP220 protein, was responsible for actin organization that controlled the membrane-distribution of aquaporin-2 (AQP2) [65]. Notably, irregularities in AQP2 have been shown to cause the abnormal kidney filtration behavior in patients with nephrogenic diabetes insipidus. Similarly, knockdown and knockout of several suspected breast cancer driver genes in a mouse mammary tissue organoid model revealed *Ptpn* and *Mll3* to be tumorigenic, the former when knocked down, and the latter under complete knockout [66].

More prominently however, application of CRISPR-Cas9 to human tissue-derived organoids has generated models of human disease that are otherwise difficult to establish in either animals or 2D cultures. Recently, two groups independently developed *ex vivo* models of human colon adenocarcinoma by using CRISPR-Cas9 to induce mutations in multiple tumor suppressor genes of intestinal organoids. The mutated organoids were shown to be self-selecting, growing in the absence of essential nutrients present in the intestinal stem cell niche, and resulted in rapid tumorigenesis when implanted in mouse models [67,68]. Similar success was observed when CRISPR-Cas9 was applied to delete the PKD1 and PKD2 genes in kidney organoids. In addition to revealing that neither gene was essential in promoting differentiation, the organoids underwent irregular cyst growth, the hallmark characteristic of polycystic kidney disease [69].

Beyond the generation of disease models, the combination of CRISPR-Cas9 with organoid cultures is potentially applicable for organoid-based therapeutics. In a proof-of-concept experiment, CRISPR-Cas9 was used to correct a mutation in the cystic fibrosis transmembrane conductor receptor (CFTR) gene in primary intestinal organoids grown from the stem cells of cystic fibrosis patients, and subsequent examination revealed that the organoids exhibited non-diseased CFTR phenotypes [70]. Although studies on this front have been much less extensive, previous investigations describe successful transplantation of *in vitro* organoids into mouse [71] and rat [72] models. Together, these advances introduce the possibility of therapeutic use, as functionally defective tissue could potentially be replaced via transplantation of primary organoids that have been genome-edited to remove diseased phenotypes.

Conclusions

The use of the CRISPR-Cas systems as genome engineering tools has catalyzed rapid development in several fields ranging from cell therapy to epigenetic studies. Taken together, we believe that the recent developments in CRISPR-Cas technology will have far-reaching applications with a variety of potential therapeutic and research-based uses. With regards to basic science, the study of mRNA and chromosomal DNA localization dynamics using modified forms of dCas9 will enable regulation of gene expression with precise spatial control within the cytoplasm, and the ability to generate models of diseased organs along with the use of pooled screens will both contribute to the identification of novel disease mechanisms and interaction pathways, and assist in drug design and the selection of therapeutic targets. Therapeutically, clinical trials that apply *ex vivo* genome engineering for immunotherapy-based treatment of cancers are already underway. Lessons learnt from these trials will provide important insights into yet unexplored areas such as the immune response to the Cas9 as well as possible off-target mutations in the human body, which in turn will establish guidelines for future *in vivo* studies. In addition, improvements in precision genome-editing techniques could potentially be used to treat several monogenic disorders independent of HDR, while further developments in RNA targeting could provide transient gene therapies, and the transplantation of genetically engineered organoids could potentially one-day be life-saving for patients with defective or damaged organs. However, while the CRISPR-Cas technology is one with immense promise, many challenges still need to be addressed before it can be used directly in humans. These include improving the efficiency and specificity of the Cas9, along with enabling precise outcome of the repair following Cas9 nuclease activity. *In vivo* delivery of the Cas9 is also a major obstacle, as is the potential immune response against both the Cas9 and the delivery vector. Should these issues be resolved, the system may move beyond being an effective biotechnology tool, and have a revolutionary impact on clinical treatments.

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** of outstanding interest

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