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To cleave or not to cleave: therapeutic gene editing with and without programmable nucleases

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In recent years, pioneering clinical studies involving the use of programmable nucleases to achieve gene editing have begun to evaluate the therapeutic potential of such approaches. For example, Sangamo BioSciences has reported successful proof-of-concept clinical studies to treat HIV infection using an engineered zinc-finger nuclease (ZFN) to inactivate the gene coding for CC chemokine receptor 5 (CCR5) —a co-receptor for HIV — in T cells *ex vivo*, as highlighted in a news article in this journal[1] (*Nat. Rev. Drug Discov.* **13**, 321–322; 2014). Initial clinical trials using CRISPR–Cas9 gene editing technology, which has rapidly become a widely used gene editing platform in biomedical research[2], were also announced in 2016.

In this article, however, we focus on an alternative group of platforms for achieving therapeutic gene editing without the use of programmable nucleases. Although such approaches were pioneered in the 1990s[3], before the advent of the nuclease-based gene editing platforms, their use and progression to clinical trials since has been held back by issues including frequencies of gene editing that are typically well below those needed for potential therapeutic applications. However, in the past 2 years, results with a diverse group of gene editing platforms — ranging from recombinogenic adeno-associated viral (AAV) vectors, to various oligonucleotide-only approaches, to nucleobase modification (TABLE 1) — have demonstrated substantially improved gene editing efficiencies[4–9], spurring renewed interest in such approaches. Moreover, these platforms do not require cleavage of the targeted DNA to achieve gene editing — a characteristic of nuclease-based mechanisms that has raised concerns about random insertion and deletions (indels) that occur in some proportion of the targeted cleavage sites and their potential for off-target genotoxicity. Here, after briefly noting some of the history of the field, we highlight recent advances with several gene editing platforms that do not use nucleases.

The early years of gene editing

Early work on gene editing in the mid-1990s using circular chemically modified RNA–DNA oligonucleotides to induce genetic changes received much attention, but ultimately failed to achieve significant levels of reproducible editing across multiple genes and cell types. The resultant controversy and scepticism about such technologies may have reduced interest in next-generation platforms using end-blocked single-stranded DNA editing oligonucleotides, which were introduced around the year 2000 (reviewed in REF. 10 and described in US patent 7,258,854; see Further information). End-blocked single-stranded editing oligonucleotides enabled reproducible editing across many cell types and target genes[10], but still failed to achieve clinically relevant editing efficiencies. Moreover, the subsequent emergence of several nuclease-based editing tools—including meganucleases, ZFNs, transcription activator-like effector nucleases (TALENs) and CRISPR–Cas9 technologies[2] — that enable robust and high frequencies of gene editing in cells, may have further reduced interest in oligonucleotide-only editing platforms.

Nevertheless, early work in the field of oligonucleotide-only editing elucidated some of the key characteristics of various platforms that are now providing higher editing frequencies, including establishing that a single DNA strand, serving as a donor template, is responsible for genome editing activity following introduction of an exogenous oligonucleotide[10]. The single-stranded oligonucleotide pairs with a complementary region within the target gene, except for the central nucleotide base(s) that is purposely constructed to create a mismatch. In one proposed mechanism of gene editing, hybridization to the target strand is thought to occur when the target strand is transiently exposed in the replication fork. Endogenous DNA repair systems recognize the artificially created mismatch and direct resolution through mismatch repair (MMR), homology-directed repair (HDR) or incorporation into a growing DNA replication fork.

Improving oligonucleotide-only platforms

Several strategies have been used to increase the frequency of gene editing by single-stranded DNA oligonucleotides. Unmodified oligonucleotides are highly susceptible to degradation by nucleases, so from the early days of the field, most groups have used terminal chemical modifications that protect against exonucleases: typically, either several terminal phosphorothioate linkages or a single terminally locked nucleic acid (LNA) linkage. However, these end-blocked DNA oligonucleotides have a projected half-life of only 10–30 minutes in cells, resulting in modest editing efficiencies[10]. It should also be noted that treatment of cell lines with phosphorothioate end-blocked editing oligonucleotides leads to nonspecific cell cycle arrest in some cell types[10]; however, alternative non-phosphorothioate end-blocks[7] or the use of peptide nucleic acid (PNA) clamps to reduce the concentration of oligonucleotides required for editing can overcome this issue[5,6] (see below).

Editing frequencies under normal conditions with end-blocked oligonucleotides hovered between 0.1% and 1%, but were increased 3- to 5-fold when the oligonucleotides were introduced into cells during their transition through S phase[10]. Greater levels of gene editing (2–4% per treatment) can also be achieved by incubating cells with 2′3′ dideoxycytidine or thymidine to slow replication forks and thus enhance the accessibility of the target to the editing oligonucleotide[10,11].

Another strategy to enhance editing efficiencies involves using oligonucleotides with internal chemical modifications that direct DNA repair to the targeted genomic DNA strand[7,11,12]. In a pioneering study[12], it was shown that oligonucleotides containing a methyl-CpG modification that exploits the endogenous base excision repair mechanism to promote the desired correction could induce ~4-fold higher levels of gene correction in mouse cells than oligonucleotides lacking the specific modification. However, the approach can only direct conversion of a thymine into a cytosine. Other studies have aimed to evade the effect of the MMR system on the editing oligonucleotide (rather than on the desired genomic DNA strand), which suppresses the efficiency of gene editing[7,11]. One study showed that 2′ sugar modifications in the oligonucleotide across from the nucleotide targeted for editing could reduce the impact of MMR, leading to ~3-fold higher editing efficiencies (~4.5%) in human cells[11]. And in a recent study[7], optimization of the editing oligonucleotide length, delivery and chemical modification pattern, including internal LNAs to evade MMR and terminal LNAs to block exonucleases, increased the efficiency of editing in mouse cells by four orders of magnitude, from $<10^{-7}$ to $>10^{-3}$.

Table 1 | **Major therapeutic genome editing approaches**

Method	DNA size	Types of edit achievable	Recognition length (nucleotides)	Frequency of target sequences	Cleavage type	Companies	Key advantages	Potential disadvantages
Protein programmable nucleases (ZFNs and TALENs)	~1–3 kb coding region	• Knockout • Knockin • Deletions • Precision point edits	~20–40	~30–200 base pairs depending on class of nuclease	Blunt or overhang	Thermo Fisher, Cellectis, Sangamo, Two Blades and Precision BioSciences	• Advanced versions with higher specificity • High editing efficiency • Targeted whole-gene (cDNA) replacement mode	• Off-target cleavage • Time to generate longer than CRISPR or exogenous editing oligonucleotides • Requires exogenous protein expression, which adds to complexity of clinical applications
CRISPR–Cas9 (ribonucleo-protein)	~3.5–4.5 kb	• Knockout • Knockin • Deletions • Precision point edits	17–20	~22 base pairs	Blunt; alternative Cas9s with overhangs	DuPont, Caribou, Editas, CRISPR Therapeutics, Cellectis and ToolGen	• Extreme ease of design • Low cost as a reagent • High targetability and multiplexing • Targeted whole-gene (cDNA) replacement mode	• Off-target cleavage • Intellectual property disputes; potentially many licences required • Requires exogenous protein expression, which adds to complexity of clinical applications
Editing by nucleobase modification	~2–5 kb	Precision point edits	~20–40	From every base pair to 22 base pairs	No cleavage	None?	• No random indels • Potential for high-efficiency point changes	• Only point changes, cannot achieve indels • Different design for each type of sequence change (for example, deamination for A to I (G)) • Early stage • Requires exogenous protein expression, which adds to complexity of clinical applications
Chemically modified editing oligonucleo-tide	Typically 20–70 nucleo-tides	Precision point edits (small indels)	20–70	Every base pair	No cleavage	ETAGEN Pharma	• Low off-target activity • Ease of design • Ease of delivery *in vivo* • Multiple treatments, cumulative editing • Established GMP manufacturing infrastructure	• Low efficiency per treatment in most cases • No targeted whole-gene (cDNA) replacement mode
Recombino-genic AAV	~5 kb genome	• Knockin • Deletions • Precision point edits	Up to several kb	Every base pair	No cleavage	• Universal Cells • LogicBIO • Homology Medicine	• No target cleavage • Ease of design • Whole-gene (cDNA) replacement mode	• Low efficiency for early formats • Requires a gene therapy vector, which adds to complexity of clinical applications

AAV, adeno-associated virus; GMP, good manufacturing practice; indels, insertions and deletions; kb, kilobases; TALEN, transcription activator-like effector nucleases; ZFN, zinc-finger nuclease.

Finally, another promising approach to enhancing editing efficiency combines triplex-forming PNAs with phosphorothiate end-blocked single-stranded editing 'donor' oligonucleotides. Using nanoparticles to deliver such PNAs and a donor oligonucleotide, ~5% correction of the F508del allele (a mutation that causes cystic fibrosis) in nasal epithelial cells at the DNA level was demonstrated in mice (corresponding to correction of ~5–10% of the cells, as there are two target copies per cell). This is a remarkable advance in the efficiency of gene editing *in vivo* using only endogenous cellular proteins to effect repair. Another very recent study by the same group also used a triplex-forming PNA combined with an end-blocked donor oligonucleotide, in conjunction with haematopoietic stem cell factor to further promote gene editing[6]. The authors obtained up to 14% edited cells in culture per treatment of haematopoietic stem cells and ~4% editing in a mouse model of beta-thalassaemia after four intravenous administrations of oligonucleotides, which resulted in long-term phenotypic correction[6].

Recombinogenic AAV

It has recently been demonstrated that much longer single-stranded donor DNA delivered by a variant of recombinant AAV vectors can also lead to editing in cell culture and in animal models without the need for exogenous nucleases[4]. Although the published efficiency of this technology is only in the 1% range, the long repair template allows for site-directed gene replacement. By directing integration of a replacement factor IX 'gene' (cDNA) to the highly expressed albumin locus with a variant of recombinant AAV in a mouse haemophilia model, 7–20% of normal factor IX was expressed in the serum, resulting in correction of the mutant phenotype without exogenous programmable nucleases[4].

Nucleobase modification

Chemical modification of a mutated nucleobase, directed by an oligonucleotide guide associated with a reactive group or base-modifying enzyme, is another method of gene editing that has been investigated since the 1990s[3]. The nucleobase modification method was first applied to mRNA editing by directing an endogenous or exogenous deaminase with an oligonucleotide guide and was subsequently proposed for use in genome editing by duplex strand-invading oligonucleotides[3]. Very recently, editing through nucleobase chemical modification in mammalian cells was demonstrated using CRISPR with a nuclease-inactivated Cas9 (deadCas9) associated with a cytidine deaminase enzyme[8]. The CRISPR guide RNA directs the deadCas9 to the site of the mutation, and the deaminase modifies the mutated cytidine to uridine. This results in highly efficient editing (up to 75%) of the mutation without chromosomal cleavage in cell culture, with a minimal frequency of insertions and deletions (indels)[8], although the deaminase also edited non-targeted cytidines in the vicinity of the targeted base[8]. Another method fuses a deaminase to a zinc finger or TALEN to obtain precise point edits in mammalian cells in culture, albeit at lower frequencies[9]. We anticipate that refinements of gene editing by nucleobase modification will achieve both high frequency and highly precise genome editing in the near future.

Outlook

Although still in its infancy, the promise of gene editing technology has attracted tremendous interest and investment, and many companies are now active in the field, pursuing a range of approaches (TABLE 1). Each mode of gene editing has distinct advantages and pitfalls for therapeutic applications, which may depend on the type of editing that is desired (TABLE 1).

The issue of off-target genome modification by editing has been noted as a particular potential concern for approaches based on nucleases, given that these involve the cleavage of genomic DNA. With regard to other approaches, although sequencing of some potential off-target sites has shown few off-target edits, the off-target activity of single-stranded oligonucleotide-based techniques or nucleobase modification needs to be studied more thoroughly. Also, for the recombinogenic AAV approach, the fact that transgene expression is dependent on correctly targeted insertion does not mean that the construct cannot insert elsewhere and induce genomic toxicity at that site. For all approaches, these concerns should be addressed rationally, as people are commonly exposed to agents and conditions that randomly mutate somatic and germline cells (such as background radiation or chemotherapeutic agents), and spontaneous random mutations occur constantly in somatic and germline cells. In this regard, genotoxicity assessment for therapeutic editing is no different from assessment of genome integrity for traditional drugs, which involves a risk–benefit analysis based on quantitative measurement of the mutation rate of treated cells or organisms compared with baseline mutation rates.

Overall, it is likely that methods with and without programmable nucleases will each have utility for the myriad of gene editing therapeutic applications. Furthermore, there may be additional benefits in editing efficiency to be gained from the combination of advances in the design of editing oligonucleotides with nuclease-based approaches.

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Competing interests statement

T.M.W., F.B. and E.B.K. own shares in ETAGEN Pharma, where T.M.W. served as chief executive officer, and F.B. and E.B.K. serve as scientific advisors. T.M.W. began a position as a technology licensing officer at the Massachusetts Institute of Technology (MIT) Technology Licensing Office on 9 November 2016, after the submission of this article, but before the final post-submission editing was completed. T.M.W. does not work on licensing of CRISPR–Cas9 at MIT and does not have any financial interest in CRISPR–Cas9 at MIT.

FURTHER INFORMATION

US patent 7,258,854: http://patentimages.storage.googleapis.com/pdfs/US7258854.pdf

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