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Therapeutic Gene Editing Safety and Specificity

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Summary

Therapeutic gene editing is an exciting new field with significant potential for the advancement of medical science across all disciplines. As with any new technology, care must be taken to address issues of safety, which in the realm of gene editing are intricately linked to the specificity of the editing tools used to cut at precise genomic targets. Safety improvements can be achieved by the thoughtful design of nucleases and repair templates, the analysis of off-target editing, and the careful utilization of viral vectors. Advancements in the understanding of DNA repair mechanisms and the development of new generations of gene editing tools allow for improved targeting of specific sequences while minimizing the risk of unintended outcomes. Now is an important time for the field of gene therapy to carefully plot a safe course for the next generation of clinical trials. Here, we review aspects of safety and specificity for therapeutic gene editing and hope to spur dialogue to advance the field.

Keywords

Gene Therapy; Safety; Specific; Gene Editing; Off-Target

Introduction

The field of therapeutic gene editing is advancing at an ever-increasing pace. As the list of diseases that can be treated or potentially cured with gene editing grows, it is imperative that we dedicate time and energy to the topics of the safety and specificity of these technologies. The following article is dedicated to a discussion of this topic in a broad sense with examples to detail how these issues impact various aspects of modifying the genetic code of our patients.

As discussed in the opening article (Kohn DB: Historical Perspective on the Current Renaissance for Hematopoietic Stem Cell Gene Therapy) of this issue, expectations have always been high for the potential of gene therapy, but early forays into its clinical application had unexpected consequences. These early setbacks in the implementation of gene therapy caused the research community and the public at large to take pause and consider the safety of this fledgling field. All involved realized that this was new and

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unexplored territory. Unexpected consequences can and do occur with new medical technologies. It is important to remember that expectations today may be higher for these pursuits than previous eras of medical exploration. Modifying genetic code is fundamentally different from the study of chemicals to kill a particular bacterial strain or slow the growth of a tumor. Rather than externally manipulating the biology of organisms or aberrant cells, we aim to alter the blueprint of the disease-associated cells to restore or modify their function. Like a surgeon pinpoints a physical defect based on accepted anatomical function and makes repairs so too are we starting with a map (the 'normal' genome) and working to correct errors. Precision and safety will be expected and demanded of our field. Trial and error will only be acceptable to a point.

Before proceeding, let us briefly discuss the stewardship of the human genome that is demanded of us. We are beginning to modify the most fundamental elements of what makes us human. This requires an enormous amount of public support and trust. While there is clearly support for the treatment and cure of genetic diseases we must look only look to history to know that these technologies can also be misused. Part of safely developing these tools is considering the consequences of their misapplication. The pursuit or even the perception of the application of gene therapy for eugenics or genetic discrimination could be devastating for the field. Care should be taken in the selection of disease targets and even in the words chosen to describe the diseases we aim to treat. Respect should be given to historic and cultural diversity. Maintaining a sense of transparency as well as being open to discussion of the practical impacts of genetic modifications are important elements to maintaining the integrity of the field of gene therapy.

Safety

Safe manipulation of the human genome is paramount to the field of gene therapy, as the intended effect of gene therapy is a permanent modification of cell function. Thus, unintended modifications that alter cell function may have long lasting consequences.

The last decade has seen the rapid introduction of new tools, including zinc finger nucleases, homing endonucleases, TAL effector nucleases and RNA-guided nucleases, that allow for the targeted modification of cellular genomes. The unifying activity for all of these tools is their nuclease activity - the ability to bind a specific sequence anywhere in the genome and introduce a DNA double strand break (DSB). Once a DSB is generated, repair occurs through one of two basic types of mechanisms: non-homologous end joining (NHEJ) or homology directed repair (HR). With enzymatically-generated double strand breaks, NHEJ will typically lead to seamless re-ligation of the break. However, NHEJ may introduce insertions or deletions at the double strand break site at appreciable frequencies which can be useful for disrupting gene expression or function, or modifying regulatory functions mediated by the targeted sequences. HR involves the repair of a DSB using a repair template with homology to the sequences flanking the cut site. This template can either be endogenous, such as from a sister chromatid, or may be exogenously introduced. Thus, in addition to simple disruption of the targeted region, HR can be used to introduce complex engineered genetic elements.

When we edit the genome with therapeutic intent, we are attempting to generate controlled genetic damage. We then rely on the native repair mechanisms of the cell to repair the damage. When we consider the safe translation of gene editing to a patient population, it is worth considering that we have been employing induced genetic damage in the form of chemotherapy and radiation for decades. The underlying principal of cancer treatment is inducing genetic damage in a fashion that is toxic to cancer cells but does not overwhelm the repair mechanisms of healthy cells. An assessment of chromosomal instability has recently been shown to help predict the survival of a patient receiving chemotherapy and/or radiation¹. Attempts to concentrate the genetic damage to sites of disease, such as administering intrathecal chemotherapy for CNS malignancies or using proton beam therapy to narrow the radiation window are steps toward tissue level specificity, but the impact on treated cells is genome wide. Side effects, both short and long term, do occur and are well known to the oncologist. Thankfully, while secondary malignancies can occur as a result of chemotherapy and radiation treatment, the majority of patients do not develop treatment related tumors. The cells either repair their genetic damage or undergo cell death in the presence of excessive damage. By comparison, the risk posed by introducing a transiently expressed, targeted endonuclease that has passed through extensive screening for off-target cutting should be significantly less dangerous. While the introduction of synthetic genetic elements by HR raises the potential for unintended consequences, the editing process and integration are similarly designed for specificity and would not be predicted to approach the magnitude of risk seen with already accepted treatments for cancer therapy.

Among potential safety concerns of gene editing, the most fundamental is the potential for the nuclease to introduce double strand breaks at unintended sites in the genome. If the precise targeted sequence or sequence sufficiently similar exists elsewhere in the genome, that site has the potential to be cleaved in addition to the intended site. When designing a targeted endonuclease, it is critical to anticipate potential off-target cleavage sites and select sequences that minimize this potential. This is most commonly carried out initially *in silico* using tools such as the PROGNOS tool for zinc fingers and TALENs (designed and maintained by the Gang Bao lab of Rice University) and the CRISPR Design tool (designed and maintained by the Feng Zhang lab of MIT)^{2,3} (<http://bao.rice.edu/Research/BioinformaticTools/prognos.html> and <http://crispr.mit.edu/>). Unlike basic primer design or sequence alignment tools, these algorithms take into account the nature of the endonuclease platform such as spacer length for TALENs and PAM sites for CRISPR and are better suited for identifying accurate off-target sites within the genome⁴. These tools offer rapid results to aid in the selection and design of potential nucleases. Once an endonuclease has been generated, *in vitro* screening has been made possible by means of SELEX (Systematic Evolution of Ligands by eXponential Enrichment)⁵. SELEX queries the propensity of the endonuclease to cleave a library of various oligonucleotides. The genome can then be probed for the presences of sequences with the highest rates of *in vitro* cleavage. Matching sequences are an excellent starting point for the search of off-target cleavage post-editing in target cells.

Screening for off-target activity is also necessarily performed following the genetic modification of target cells using the intended manufacturing process for cells to be engrafted in patients. Sequencing at *in silico* predicted off-target cut sites can reveal if

mutation has occurred at these specific loci. Mutation events at sites not included in the *in silico* prediction as well as non-mutational on-target cleavage repairs will be missed by this technique. Genome wide off-target editing analysis has been analyzed either by detection of repair template integration or ChIP-seq (chromatin immunoprecipitation followed by high-throughput sequencing) analysis. Repair templates delivered by non-integrating viruses should only be detectable at sites of endonuclease activity, therefore primers specific to the repair template but not present in the genome at large can be used to map sites of nuclease activity⁶. ChIP-seq can be used to demonstrate sites of nuclease binding to the genome, but does not necessarily indicate cleavage events have occurred⁷. While both methods offer insight into potential off-target cleavage sites, neither offers comprehensive coverage of the genome or quantification of the likelihood of such events occurring. A hybrid approach called GUIDE-seq has also been described that uses blunt ended double-stranded oligodeoxynucleotide (dsODN) integration at cut sites followed by next generation sequencing⁸. DNA double strand breaks either on-target or off-target also have the potential to result in translocation events that can be difficult to detect. A high-throughput genome-wide translocation sequencing (HTGTS) technique has been described that aims to both predict and monitor for these events at specific on-target or off-target sites⁹.

Care should also be taken to consider the anticipated and unanticipated biologic impact of the intended gene modification when designing a targeted endonuclease. Unintended impacts can range from decreased cell survival and function to clonal proliferation and oncologic transformation. Gene therapies have been proposed that target intronic sequence, repressor as well as promoter and enhancer elements, transcription factors and the insertion of synthetic constructs to name a few¹⁰. Therapies that rely on the targeted disruption of a transcription factor must consider not just the impact on the gene causing the undesirable phenotype, but genes in other pathways as well. The introduction of synthetic gene elements by homologous recombination requires further scrutiny to monitor for correct insertion and the potential impact on nearby genes both at the intended and off-target integration sites. One approach to mitigate the potential risk of oncologic transformation is the introduction of a suicide gene that can be activated to eradicate the offending population if needed¹¹.

Safety considerations should also extend beyond the direct safety to the patient and include lab staff, clinical staff and direct contacts of the patient. Lab staff safety starts with the proper handling of cells and reagents based on appropriate biosafety level assignment to the reagents being used. Transfection of mRNA or DNA constructs by electroporation poses little risk to the lab staff member. The use of viral elements such as AAV, IDLV or others increases the required safety precautions. Both the nature of the virus and the payload being delivered should be evaluated for the potential to cause harm if lab staff members are exposed. Viral carriers that introduce HR templates alone are arguably less dangerous than those containing nuclease elements. Viruses with the potential to integrate into the genome or infect epithelial cells should be handled with greater care. The risk to clinical staff including nurses and doctors should be relatively minimal in most cases. Certainly, if the gene therapy takes place in the lab (such as editing hematopoietic stem and progenitor cells) the cells that are infused into the patient are isolated from the clinical staff and pose little risk if an exposure should occur. The risk posed by gene therapy involving the intravenous or direct tissue inoculation with an engineered virus will need to be considered for each

protocol. Aerosolization or unintentional needle sticks of infectious elements have the potential to harm clinical staff and a plan to respond to such exposures will need to be part of any gene therapy protocol. Family and close contacts of the patient will need to be aware of any potential shedding of engineered virus by the patient. Fortunately, in the vast majority of cases, the risk will be minimal.

An important topic of debate concerning therapeutic gene editing is the potential modification of germline cells. For families where one or more members carry a pathogenic genetic trait, ridding their lineage of that trait, even if they would not be able to change their own clinical outcome, is a reasonable aspiration. At our current level of understanding of gene editing, there are both known and unknown risks that render attempts at germline gene editing ethically questionable even for the elimination of traits with unequivocal clinical benefit. First and foremost, we do not currently understand our genetic tools well enough to know that we can wield them for a beneficial outcome without unknowingly causing damage. Off target cleavage events are known potential sequelae of gene editing and even with current sequencing technology, it is not possible to know that an attempt at beneficial editing has not also resulted in accompanying detrimental genetic alterations. Further issues would arise from any attempt at non-therapeutic genetic enhancement where the clinical benefit of modifying a genetic trait is less clear. While modifying a trait or combination of genetic traits in a patient may achieve a desirable phenotypic outcome, we cannot be certain that the impact will be the same in the genetic milieu of their offspring. With these uncertainties, while a patient can consent to undertake the risk of modifying their own genome, it is not yet clear that those decisions can or should be propagated to their progeny through germline editing. Based in part on these issues, NIH guidelines currently prohibit the use of federal funds for research that aims to alter the germline in either mature adults or human embryos, although it is worth noting that a recent report from the National Academy of Sciences seems leave the door open for such a possibility in the future^{12,13}. Due to the technical and ethical issues associated with germline modification, current practice is that any potential modification of germline cells must be carefully considered and arguably avoided.

Medical monitoring in both the short and long term following a gene therapy procedure must also be carefully planned in advance. The concept of the 'medical home' for gene therapy patients has not yet been well established. It is unlikely that there will be a gene therapy division in most hospital settings but rather care will be carried out by the specialty service caring for the underlying diagnosis. A SCID patient will likely be treated by an immunology department, a sickle cell patient by hematology and so on. The challenge is that if we achieve the curative treatment we hope for, the patient may be lost to follow-up as their symptoms resolve. This is a particularly important issue in the near future, as due to our relative inexperience with gene therapy and gene editing, it is advisable (and currently an FDA requirement) to monitor gene therapy patients over long periods of time for the development of therapy related complications. Ensuring that detailed information on the exact genetic manipulation the patient undergoes follows them later in life in some form of medical record will be essential so that any adverse events could be related to general or specific aspects of gene therapy or gene editing. If the potential for germline modification

exists, multi-generational access to this data may also be advisable but adds a high degree of medico-legal complexity.

Specificity

Critical to the success and safety of targeted endonucleases and other forms of gene therapy is the degree of specificity that can be achieved. The random integration events that occur with retroviral gene delivery demonstrate the danger of gene therapy in the absence of high degrees of specificity. New generations of targeted endonucleases including zinc finger nucleases, TALENs, and CRISPR/Cas9 each have unique mechanisms for binding to specific genomic sequences to induce targeted cleavage events. Zinc fingers and TALENs utilize nucleotide specific protein motifs and CRISPR/Cas9 relies on Watson-Crick base pairing of an RNA guide to recognize specific genomic targets. Here, we will consider the unique features of each of these approaches for engineered specificity.

Zinc fingers were some of the first sequence specific tools used for gene editing¹⁴. Zinc fingers are protein motifs that function as transcriptional regulators by recognizing 3 base pair sequences. Libraries of zinc fingers have been generated that target a wide array of 3-bp sequences. Series of zinc fingers (typically 3–6 per monomer) can be connected to yield sequence specificity of 9–18 bp¹⁵. Additional specificity is achieved by nature of linking the obligate heterodimer restriction enzyme *FokI* to two zinc finger constructs flanking the intended cut site. As individual zinc finger-*FokI* monomers are not capable of cleaving without a partner, the target sequence must match not only the sequences encoded by both zinc finger constructs, but also be in the correct orientation and separated by roughly the same number of bases required to align the *FokI* elements. The chance of all elements being present in regions of the genome other than the intended cleavage site becomes exceedingly rare.

Not long after the first reported use of zinc finger based constructs for gene editing, TAL effector nucleases (TALENs) were developed as an alternative mechanism for targeting specific sequences. Transcription activator-like effectors (TALEs) were identified as DNA binding proteins expressed by plant pathogens as a means of avoiding host defenses via genetic manipulation¹⁶. Unlike zinc fingers which recognize 3 bp sequences, an individual TALE subunit (referred to as a repeat variable diresidue, or RVD) recognizes a single DNA base pair. The TALE subunit is a 34 amino acid protein element that is identical except for amino acids 12 and 13 that impart specific recognition of different DNA bases (i.e. A, T, C, or G). Individual TALE subunits can be combined in series (typically of 15–20 subunits) to create a helical structure that traces the major groove of a specific DNA sequence¹⁷. Like zinc fingers, TALENs are generated by linking TALE subunits to the *FokI* endonuclease and are also used in heterodimeric pairs which further increases specificity¹⁸.

More recently, the CRISPR/Cas9 system has become a major focus of the therapeutic gene editing field. The system is based on a prokaryotic immune defense system that stores short segments of invading virus or plasmid DNA in a form of molecular memory. These sequences are expressed as guide RNA molecules that target the CRISPR-associated endonuclease (Cas) to the same sequence in the offending virus and introduce double

stranded breaks¹⁹. The sequence specificity is imparted by Watson-Crick base pairing of the guide RNA to the target DNA sequence. This mechanism has been adapted for use in eukaryotic cells primarily using an optimized Cas9 endonuclease²⁰. What makes the CRISPR/Cas9 system so appealing is the relative ease of generating ~20-bp guide RNA sequences. Unlike zinc finger nucleases or TALENs, CRISPR/Cas9 functions as a monomer and thus the specificity relies on the guide sequence binding. The ability to rapidly design and generate guide RNA to test potential target cleavage sites make the CRISPR/Cas9 system particularly appealing for screening guides toward multiple targets, or for editing procedures that require cleavage of multiple targets.

Each of these techniques is based on the specific binding of a targeted genomic sequence. Multiple attempts are underway to improve the specificity of each platform to minimize off-target cleavage and improve safety. One technique involves the directed mutagenesis of *FokI* to generate obligate heterodimers which reduces the incidence of homodimer cleavage events²¹. Altering the linker sequence between the *FokI* and the zinc finger has also been shown to increase the specificity of zinc finger pairs²². Further specificity can be gained by joining the TALE subunits to a site-specific meganuclease in place of the *FokI* to form 'megaTALs', but only in sites with favorable characteristics²³. An expanded set of RVDs has been generated and analyzed to achieve improved TALEN specificity²⁴. The specificity of the CRISPR/Cas9 system has been a topic of much debate. Some report evidence of decreased specificity resulting in double strand breaks at sites with only 15 of 20 base pair matches or large chromosomal deletions^{25,26}. Others report little or no off-target cleavage using CRISPR/Cas9 at other sites leaving open the possibility of site or sequence dependent specificity. A recent effort to overcome this potential limitation is the re-engineering of *Streptococcus pyogenes* Cas9 (SpCas9) to reduce off-target cleavage events²⁷, although an unintended effect of many types of specificity engineering may be an associated reduction in on-target cleavage efficiency which may limit utility.

While the specificity of various targeted endonuclease technologies is based largely on sequence targeting motifs within the construct, there are other factors that contribute to the generation of off-target cleavage. Two important factors are the level and duration of time the genome is exposed to the endonuclease. Even a highly specific cutting tool has the potential for a low rate of off-target editing that can be compounded by particularly high level or prolonged expression. As the field has moved away from lentiviral vectors due to the risk of genomic integration, viral delivery with adeno-associated virus (AAV) and other non-integrating viral vectors has emerged as a mechanism for delivering endonucleases to target cells. Depending on the infectivity of the virus, the activity of the expression cassette delivered and the rate of clearance of each from the cell, endonuclease activity can persist for days. While this may increase overall cutting efficiency at the target site, it also has the potential to increase off-target genomic damage. One means of limiting the exposure time of the genome to an engineered endonuclease is to deliver it in RNA form. The transfection of mRNA directly into target cells is possible *ex vivo* in cells such as hematopoietic stem cells. Zinc fingers, TALENs and Cas9 can all be delivered in mRNA form²⁸. This limits the nuclease exposure time to the persistence of the mRNA and translated protein^{18,29}. CRISPR guide RNA and Cas9 protein can also be complexed as a ribonucleoprotein (RNP) for direct electroporation which similarly achieves limited duration.

Another aspect of specificity relates to the viral delivery of the engineered nuclease, particularly if delivered as a systemic infusion for in vivo genome editing applications. As described in other chapters, AAV has become a major player in the implementation of gene therapy as a means to deliver genes as well as editing components. At least 13 naturally occurring AAV serotypes have been studied and found to have at least partial affinity for various organs. AAV2 has been found to exert natural tropism for skeletal and vascular smooth muscle, hepatocytes and neurons and AAV8 has a high affinity for hepatocytes as well³⁰. Work is also underway to conduct directed evolution of the AAV virus to decrease immunogenicity, increase tissue specific tropism, overcome cellular barriers and increase packaging capacity³¹. Modified AAV plasmids and vectors have been generated that co-deliver Cas9 and guide RNA and can also preferentially target specific cell types³². Tissue specificity of a viral vector delivery system only adds to the overall specificity of a gene editing strategy.

As discussed previously, one of the two major endogenous repair mechanisms following the introduction of a DSB is template guided homologous recombination (HR). Repair templates contain homology arms on both the 5' and 3' ends of the construct that correspond to the sequence flanking the endonuclease cut site. Additional genetic content can be encoded between the arms and incorporated into the cut site. The homology arms are typically hundreds of base pairs long making it unlikely that the template will insert anywhere but on-target cut sites. Editing strategies that rely on HR template integration must plan for a subset of cells to undergo NHEJ without template integration and the impact of these events should be reviewed for potential deleterious effects. Worth mentioning here is the importance of ensuring that repair templates do not share homology with the full target site rendering them cleavable. An analysis of the frequency and impact of SNPs both at the putative cut site as well as within the homology arm recognition site is an important aspect to applying an HR based gene therapy to a patient population. HR template integration is a powerful component of targeted gene editing and further adds to the specificity of these approaches.

Summary/Discussion

Learning from the challenges of the first gene therapy trials, a large emphasis has been placed on improved specificity to achieve safety. The new generation of targeted nucleases has made the possibility of precise genetic manipulation a reality. The purpose of this review is not to endorse one particular platform over another. Each has their merits and all have the potential for safe and effective therapeutic application. Importantly, large-scale cross-platform comparisons of safety and specificity are of limited utility. Rather, safety and specificity must be assessed for each individual editing process due to multiple potential variables including target site characteristics, nuclease manufacturing and delivery, editing procedure and cell handling. Ideally, specificity and safety of each new therapy should be addressed early in the design phase and reassessed as implementation proceeds. It is important to remember that the field of gene therapy and gene editing are still in the early stages and prudence is warranted to diligently pursue the highest possible standards of safety. The ability to sequence and modify the human genome will help shape the legacy of modern medical science. How we proceed in studying and implementing this incredible new

ability has long-ranging implications for the integrity and public trust for both the practice of science and medicine.

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References

1. Blaese RM, Culver KW, Miller AD, et al. T Lymphocyte-Directed Gene Therapy for ADA– SCID: IniLal Trial Results After 4 Years. *Science*. 1995; 270(5235):475–480. [PubMed: 7570001]
2. Fine EJ, Cradick TJ, Zhao CL, Lin Y, Bao G. An online bioinformatics tool predicts zinc finger and TALE nuclease off-target cleavage. *Nucleic Acids Res*. 2014; 42(6):e42–e42. [PubMed: 24381193]
3. Optimized CRISPR Design
4. Hsu PD, Scott DA, Weinstein JA, et al. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol*. 2013; 31(9):827–832. [PubMed: 23873081]
5. Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science*. 1990; 249(4968):505–510. [PubMed: 2200121]
6. Petek LM, Russell DW, Miller DG. Frequent Endonuclease Cleavage at Off-target Locations In Vivo. *Mol. Ther*. 2010; 18(5):983–986. [PubMed: 20216527]
7. O'Geen H, Henry IM, Bhakta MS, Meckler JF, Segal DJ. A genome-wide analysis of Cas9 binding specificity using ChIP-seq and targeted sequence capture. *Nucleic Acids Res*. 2015; 43(6):3389–3404. [PubMed: 25712100]
8. Tsai SQ, Zheng Z, Nguyen NT, et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat. Biotechnol*. 2015; 33(2):187–197. [PubMed: 25513782]
9. Chiarle R, Zhang Y, Frock RL, et al. Genome-wide translocation sequencing reveals mechanisms of chromosome breaks and rearrangements in B cells. *Cell*. 2011; 147(1):107–119. [PubMed: 21962511]
10. Maeder ML, Gersbach CA. Genome-editing Technologies for Gene and Cell Therapy. *Mol. Ther. J. Am. Soc. Gene Ther*. 2016; 24(3):430–446.
11. Jones BS, Lamb LS, Goldman F, Di Stasi A. Improving the safety of cell therapy products by suicide gene transfer. *Front. Pharmacol*. 2014; 5
12. Collins FS. Statement on NIH funding of research using gene-editing technologies in human embryos. *Natl. Inst. Health NIH*. 2015
13. Committee on Human Gene Editing: Scientific, Medical, and Ethical Considerations, National Academy of Sciences, National Academy of Medicine, National Academies of Sciences, Engineering, and Medicine. *Human Genome Editing: Science, Ethics, and Governance*. Washington, D.C.: National Academies Press; 2017.
14. Bibikova M, Golic M, Golic KG, Carroll D. Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. *Genetics*. 2002; 161(3):1169–1175. [PubMed: 12136019]
15. Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet*. 2010; 11(9):636–646. [PubMed: 20717154]
16. Fujikawa T, Ishihara H, Leach JE, Tsuyumu S. Suppression of Defense Response in Plants by the avrBs3/pthA Gene Family of *Xanthomonas* spp. *Mol. Plant. Microbe Interact*. 2006; 19(3):342–349. [PubMed: 16570663]
17. Cermak T, Doyle EL, Christian M, et al. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res*. 2011; 39(12):e82–e82. [PubMed: 21493687]
18. Wright DA, Li T, Yang B, Spalding MH. TALEN-mediated genome editing: prospects and perspectives. *Biochem. J*. 2014; 462(1):15–24. [PubMed: 25057889]

19. van der Oost J, Jore MM, Westra ER, Lundgren M, Brouns SJJ. CRISPR-based adaptive and heritable immunity in prokaryotes. *Trends Biochem. Sci.* 2009; 34(8):401–407. [PubMed: 19646880]
20. Mali P, Yang L, Esvelt KM, et al. RNA-guided human genome engineering via Cas9. *Science.* 2013; 339(6121):823–826. [PubMed: 23287722]
21. Miller JC, Holmes MC, Wang J, et al. An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat. Biotechnol.* 2007; 25(7):778–785. [PubMed: 17603475]
22. Händel E-M, Alwin S, Cathomen T. Expanding or Restricting the Target Site Repertoire of Zinc-finger Nucleases: The Inter-domain Linker as a Major Determinant of Target Site Selectivity. *Mol. Ther.* 2008; 17(1):104–111. [PubMed: 19002164]
23. Boissel S, Jarjour J, Astrakhan A, et al. megaTALs: a rare-cleaving nuclease architecture for therapeutic genome engineering. *Nucleic Acids Res.* 2014; 42(4):2591–2601. [PubMed: 24285304]
24. Miller JC, Zhang L, Xia DF, et al. Improved specificity of TALE-based genome editing using an expanded RVD repertoire. *Nat. Methods.* 2015; 12(5):465–471. [PubMed: 25799440]
25. Fu Y, Foden JA, Khayter C, et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* 2013; 31(9):822–826. [PubMed: 23792628]
26. Cradick TJ, Fine EJ, Antico CJ, Bao G. CRISPR/Cas9 systems targeting β -globin and CCR5 genes have substantial off-target activity. *Nucleic Acids Res.* 2013; 41(20):9584–9592. [PubMed: 23939622]
27. Slaymaker IM, Gao L, Zetsche B, et al. Rationally engineered Cas9 nucleases with improved specificity. *Science.* 2016; 351(6268):84–88. [PubMed: 26628643]
28. Hendel A, Bak RO, Clark JT, et al. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat. Biotechnol.* 2015 advance online publication.
29. Pruett-Miller SM, Reading DW, Porter SN, Porteus MH. Attenuation of Zinc Finger Nuclease Toxicity by Small-Molecule Regulation of Protein Levels. *PLoS Genet.* 2009; 5(2)
30. Srivastava A. In vivo tissue-tropism of adeno-associated viral vectors. *Curr. Opin. Virol.* 2016; 21:75–80. [PubMed: 27596608]
31. Kotterman MA, Schaffer DV. Engineering adeno-associated viruses for clinical gene therapy. *Nat. Rev. Genet.* 2014; 15(7):445–451. [PubMed: 24840552]
32. Senis E, Fatouros C, Große S, et al. CRISPR/Cas9-mediated genome engineering: An adeno-associated viral (AAV) vector toolbox. *Biotechnol. J.* 2014; 9(11):1402–1412. [PubMed: 25186301]

Key Points

- Safety of gene editing is closely tied to specificity.
- Careful design of gene editing tools can improve specificity and thus safety.
- A high degree of specificity is possible with the new generation of targeted nucleases.
- Assessing the impact of gene therapy tools during their design, study and clinical use is essential.