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doi:10.1038/mt.2015.54

Genome Editing Technologies: Defining a Path to Clinic

Genomic Editing: Establishing Preclinical Toxicology Standards

Bethesda, Maryland
10 June 2014

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Recently developed genomic editing technologies have the potential to be powerful tools for gene therapy because of their ability to inactivate genes, correct mutated sequences, or insert intact genes. While the genomic editing field is advancing at an exceptionally rapid pace, there remain key issues regarding development of appropriate preclinical assays to evaluate off-target effects and establish safety. In order to begin a dialogue on these issues, the National Institutes of Health (NIH) Office of Science Policy, in collaboration with several NIH-funded investigators and the NIH Recombinant DNA Advisory Committee, organized a workshop on 10 June 2014, in Bethesda, Maryland, to provide a forum to educate the scientific and oversight communities and the public on different genome editing technologies, clinical experiences to date, and the preclinical assays being developed to examine the precision of these tools and their suitability for clinical application.

Targeted genome modification by designer nucleases is an emerging technology that can be used to investigate gene function and could also be used to treat genetic or acquired diseases. A wide range of genome alterations has been achieved by these nucleases, including localized mutagenesis, local and dispersed sequence replacements, large and small insertions and deletions, and even chromosomal translocations. The nuclease approach to targeted genome editing has been applied successfully to more than 50 different organisms, including crop plants, livestock, and humans.[1]

Recently developed genome editing technologies such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases, and clustered regularly interspaced short palindromic repeats (CRISPR) are being investigated as promising tools for human gene therapy. ZFNs are the first class of nucleases to have reached the clinic in phase I trials for HIV.[2] One key issue that will have to be addressed as these technologies move into clinical trials is whether technology-specific preclinical evaluations are available that can establish safety. Although these are targeted editing tools, their precision, and specifically the degree to which there are off-target actions and the clinical implications of such activity are important questions for the field.

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Overview of genome editing technologies

The fundamental process common to all of these technologies is the use of nucleases to make site-specific double-stranded breaks (DSBs) in the genome. Several approaches to genome editing have been developed; this summary describes them briefly.

ZFNs. ZFNs are the most clinically advanced nuclease platform. Each zinc-finger consists of ~30 amino acids that fold into a conserved ββα configuration.[3] Each "finger" recognizes about three or four base pairs of DNA using at least six amino acids through contacts between specific residues in the second α-helix, also known as the "recognition helix" terminus. Three to six individual fingers can be linked to enable construction of arrays that recognize longer sequences of 9–18 base pairs (bp). Of note, 18 bp of DNA sequence can confer specificity within 68 billion base pairs of DNA. Further specificity can be engineered by changing critical residues within the recognition helices. In addition, individual fingers in an array can potentially interact and make base-specific contacts into the sequences recognized by adjacent fingers, and optimization of binding can therefore be potentially altered by interactions between individual fingers. The nuclease domain of the zinc-finger is derived from the C-terminus of the FokI restriction endonuclease. FokI only cuts DNA when it dimerizes, so two sets of zinc-fingers are required (see **Figure 1a**). The natural enzyme generates a 5′ overhang. However, one can engineer these enzymes such that within the dimer, one side of the nuclease domain is catalytically active while its twin is catalytically inactive,

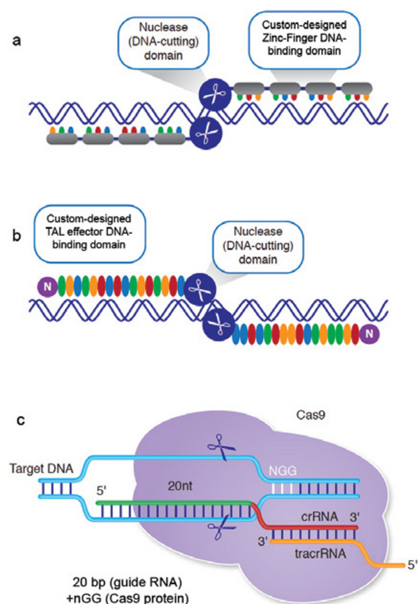

Figure 1 Nuclease site recognition features (**a**) Zinc-finger nuclease dimer: recognition sites 9–18 bp × 2. (**b**) TAL effector nuclease (TALEN): recognition sites 12–20 bp × 2, spacer 12–20 bp. (**c**) RNA-guided endonuclease CRISPR/Cas9. Courtesy of Matthew Porteus

which will generate a single-stranded nick rather than a DSB.

TALENS. TALENs are similar in architecture to ZFNs except that they use a different DNA-binding domain. They consist of arrays of single protein modules that each recognize a single DNA base pair and that are derived from transcription activator-like effectors (TALEs), factors encoded by plant pathogenic bacteria[4] (see **Figure 1b**). Each of these modules is about 34 amino acids long, and they are nearly identical except for the identities of amino acids at positions 12 and 13, which together are known as the "repeat variable di-residue". To engineer DNA-binding domains with novel DNA-binding specificities, individual TALE repeats are assembled into an array that is designed to recognize the target DNA sequence. Although the single-nucleotide specificity of TALE repeats potentially offers greater design flexibility than do zinc-fingers, their highly repetitive nature presents technical challenges in assembling DNA-encoding arrays of these domains. Different strategies have been developed to facilitate rapid assembly of DNA-encoding TALE repeats, including the "Golden Gate" assembly method[5] and a system called FLASH.[6] TALE repeat arrays that recognize 13–20 bp can be constructed. The nuclease domain used in TALENs is also from FokI; however, in contrast to ZFNs and for reasons that are not yet understood, TALENs cannot be manipulated to create nicks rather than DSBs.

CRISPR/CRISPR-associated (Cas) protein 9. CRISPR/Cas9 is distinct from the previous engineered endonucleases in that it uses an RNA-guided system to perform genome editing. This platform, derived from a bacterial innate immune system, was described relatively recently, but progress on its development has been rapid. It has captured considerable attention due to the relative ease of engineering its RNA-based targeting component. In bacteria, type II CRISPR systems process foreign sequences from invading phages or plasmids into small segments that are then introduced into the CRISPR array, which contains the regularly interspaced palindromic repeats. These snippets of foreign DNA become templates for CRISPR RNA (crRNA), which now contains a variable sequence from the invading DNA. This crRNA then hybridizes with a *trans*-activating RNA (tracrRNA), and the RNAs form complexes with the Cas9 protein. The next time this foreign sequence is detected, it is cleaved and degraded (see **Figure 1c**).[7]

Recognition of the target DNA sequence is mediated between the genomic DNA target and by a 20-nucleotide sequence in the crRNA. Another feature of this system is that the Cas9 protein is directed to cleave the complementary target-DNA sequence if it is adjacent to a short sequence known as the protospacer adjacent motif (PAM). The PAM sequence commonly used from *Streptococcus pyogenes* has the sequence [N]GG, although [N]AG can also be used. Many bacteria have the Cas9 system, but not all of them possess the same PAM sequences, providing some additional variability. In addition, mutations in the PAM sequence will prevent the Cas9 protein from causing a break at that site. In 2012 it was shown that the crRNA and the tracrRNA can be combined into a single RNA molecule known as a guide RNA (or gRNA) that can still engage the Cas9 protein.[8] As discussed later, gRNAs recognize 20-bp target sites but can also recognize off-target sites bearing up to six mismatches and/or bulges. Similar to ZFNs, it is possible to make a nick rather than a break with the CRISPR/Cas9 system. However, unlike the ZFNs and TALENs, the double-strand cut made by CRISPR/Cas9 leaves a blunt end.

Homing endonucleases (meganucleases). These are natural proteins called endodeoxyribonucleases that recognize long (>12 bp) DNA sequences with high specificity. Described as genetic parasites, they target the recognition site in an allele, make a break in that allele, and target the transfer of their own protein-coding sequence into that allele by homologous recombination. They generate a DSB with a four-base 3′ overhang, which is proposed to be a natural substrate for the homologous recombination machinery. Initial genome editing manipulations were done using the I-CreI and the I-SceI endonucleases. Engineering these nucleases is difficult primarily because both recognition and enzymatic activity are intertwined within the protein, often making it difficult to alter one without having an effect on the other. However, these nucleases are small and can be easily contained in commonly used vectors. In sum, they offer potentially high specificity, but barriers to their engineering are high.[9]

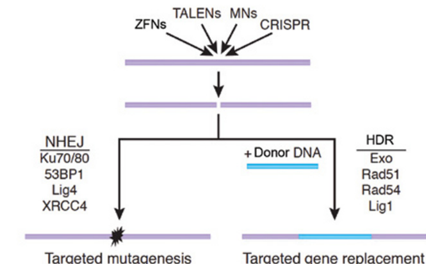

Figure 2 Mechanisms of DNA repair after targeted cleavage.HDR, homology-dependent repair; NHEJ, nonhomologous end joining. Courtesy of Dana Carroll.

Impact of double-stranded breaks

The mechanism unifying each of these technologies is the ability to make targeted DSBs in genomic DNA. The outcome of targeted cleavage depends on cellular repair pathways of DSBs, which are potentially lethal to the cell unless they are repaired quickly. The principal mechanisms of repair are nonhomologous end joining (NHEJ) and homology-dependent repair (HDR) (see **Figure 2**). NHEJ often results in sequence changes at the cut site, most commonly variable-length insertions or

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deletions, referred to as "indels," which can be detected through sequencing or mismatch cleavage assays that use enzymes such as CelI or T7 endonuclease I and more recently a new method called TIDE (tracking of indels by decomposition).[10]

There are subtle differences among the NHEJ products generated by each of the above nucleases. Small deletions and insertions are common in NHEJ mutations, but the average deletion size is somewhat larger with TALENs and ZFNs compared to CRISPRs, and insertions are somewhat more frequent with ZFNs.[1] Rarely are insertions large enough that the source of the inserted sequences can be identified.[11] Such findings highlight that the mechanisms of DSB repair are still not completely understood.

HDR can incorporate user-provided sequence changes from a donor DNA. NHEJ dominates in almost all systems, but there may be ways to shift the balance toward HDR through downregulation of enzymes that are involved in the NHEJ pathway or modulating the stage of the cell cycle. In addition, altering engineered nucleases to produce a single-stranded nick rather than a DSB will favor HDR, but at an overall lower absolute frequency of repair.[12,13]

If the goal is to incorporate new genetic material rather than just to disrupt a particular sequence, the nature of the donor DNA will also influence the success of the approach. With long double-stranded donor DNAs, successful incorporation requires several hundred base pairs of homology on both sides of the nuclease-induced break.[11,14] Short single-stranded donor DNAs can also be used, but such templates would be limited to applications requiring small changes close to the DSB, such as correction of point mutations. In some systems (e.g., *Drosophila*), sequences throughout the length of a long donor can be captured at the target, but in other cases (e.g., cultured mammalian cells) only sequences close to the break are routinely incorporated, although sequences several kilobases away can occasionally be incorporated.[15,16] Further knowledge of the activities that control this feature, known as "conversion tract length," is needed as the field moves toward gene insertion applications.

Improving specificity through design

Because genome editing is directed at specific sites, this technology offers greater precision compared to other approaches to long-term gene modification, e.g., delivery of a gene by an integrating viral vector. Most integrating viral vectors have a largely random integration pattern. However, despite the elegant precision that these editing tools offer, off-target effects are likely, depending upon the construct and the length of the target site. Ideally, the insertions, deletions, inversions, and translocations that may result from NHEJ or HDR at these off-target sites must be minimized before moving to the clinic.

Improving on the natural design. much has been learned regarding how to use these tools to target specific sequences, and the designs of these naturally occurring tools are being modified to improve the specificity. At the workshop, Keith Joung reviewed his research with different platforms—ZFNs, TALENs, and CRISPR/Cas9 nucleases—in which he generally has seen stronger binding to on-target sites compared with off-target sites, but there is still considerable activity at off-target sequences. Focusing on the CRISPR/Cas9 platform, he examined the off-target effects of first-generation CRISPR/Cas9 agents that were directed by six different gRNAs and demonstrated that there were a number of off-target sites (harboring as many as five mismatches relative to the on-target site) and that the rate of mutagenesis at these sites could be as high as that seen at the on-target site.17 This analysis was done across different cell types, and the mutations sometimes fell within the coding sequences of the genome.

This finding has led to the development of second-generation CRISPR/Cas9 agents with modifications to increase specificity. Intuitively one might conclude that increasing the length of the binding site for the nuclease would increase specificity, but Dr Joung found that for the CRISPR/Cas 9 system, specificity can be improved by truncating the 5′ end of the gRNA by as many as three nucleotides. This truncation generally does not impair the ability of a gRNA to direct on-target site cleavage but appears to make the gRNA/Cas9 complex more sensitive to mismatches and therefore reduces off-target site cleavage.[18] This truncation approach does not work for all gRNAs but appears to work well for the vast majority of gRNAs tested to date. Another strategy is to combine the specificity from different platforms. For example, it is possible to combine the dimerization-dependent FokI nuclease used in ZFNs and TALENs with the CRISPR/Cas9 system. By fusing the FokI nuclease to a catalytically inactive Cas9, one can use the gRNAs to direct binding but require dimerization for cleavage.[19,20] In one experiment in which five off-target sites were previously observed with the use of a specific gRNA, deep sequencing was used to confirm that the frequency of indel mutations induced by the dimeric CRISPR (or RNA-guided FokI nuclease) was not greater than background at all five off-target sites, indicating the ability of this platform to eliminate activity at the off-target sites of a single gRNA. This hybrid platform provides an increase in the length of the binding site by utilizing both gRNAs in a dimeric configuration and reduction in potential off-target sites.[19] Interestingly, in that experiment the authors also failed to see detectable evidence of off-target activity for these dimeric RNA-guided FokI nucleases, even at the most closely mismatched sites in the genome.

Another approach being explored is to mutate the Cas9 protein so that it will only create a nick rather than a DSB. By then pairing two such mutated Cas9 proteins with gRNAs that are offset, one can create DSBs while increasing the specificity of cleavage and significantly reducing off-target breaks.[21] Essentially this approach attempts to increase the specificity in the same way that dimerizing nucleases does but with the caveat that monomeric nickases still have the potential to induce mutations.[19] Further research is needed to determine whether these strategies can be combined to enhance specificity further.

A recent study describes a method for gRNA design that significantly enhanced the frequency of genome editing by Cas9 in *Caenorhabditis elegans*. The key innovation was to design gRNAs with a GG motif at the 3′ end of their target-specific sequences. All guides designed for all targets supported robust genome editing, both imprecise NHEJ events and precise,

template HDR events.[22]

Dr Scharenberg reviewed work he has done with Cellectis Therapeutics and Seattle Children's Research Institute combining a meganuclease, or homing endonuclease, with a TAL array.[23] The goal is to combine the high binding specificity of the TAL with the high cleavage specificity of the meganuclease. By fusing a site-specific meganuclease to a TAL array that binds adjacent to the meganuclease target site (thus tethering the meganuclease adjacent to its desired target site), one can increase cleavage activity at that target site and minimize off-target activity, as tethering will not occur at off-target sites lacking an adjacent sequence capable of being bound by the TAL array. The homing endonuclease has to be engineered, but the specificity can be improved through the engineering of the TAL, which is easier.

Scharenberg and colleagues have explored this construct (called a megaTAL) in T cells, in which the goal is to engineer the T cell to express a specific chimeric antigen receptor and at the same time disrupt the native T-cell receptor alpha (TCRα). This approach would allow for allogeneic designer T cells, currently being tested for a number of oncology applications, to function without the risk of graft-vs.-host disease (GVHD). The activity and specificity of the meganuclease targeting TCRα was assessed both with and without fusion to a TAL array that could bind a DNA sequence upstream from the cleavage site for the TCRα-specific meganuclease. Cleavage of the TCRα gene using the meganuclease alone was ~1.6% but was increased by 20-fold with the megaTAL construct. The megaTAL was then tested with cotransfection of each nuclease with Trex2, a 3′ endonuclease that can trim back the 3′ overhangs that homing endonucleases make, thereby markedly accentuating the generation of indels. Addition of Trex2 further increased the rate of disruption, yielding rates of TCRα disruption consistently exceeding 70%.[23]

This research underscores that the specificity of these tools could be enhanced further by combining them to take advantage of their respective specificity and ease of engineering. Nonetheless, an assessment of safety and specificity using preclinical assays will still be necessary, no matter which nuclease platform is utilized

Identifying safe harbors. An alternative strategy to achieve safe, targeted gene delivery and limit off-target activity is to identify sites in the human genome that are at minimal risk of causing insertional oncogenesis upon integration of foreign DNA, while being accessible to a highly specific nuclease with minimal off-target activity. Such "genomic safe harbors" may be extragenic sites that are remote from a gene or genomic regulatory sequence, or intragenic sites (within a gene) whose disruption is deemed to be tolerable. Drawing from human clinical trial data on integration sites for retroviral and lentiviral vectors, several researchers[24,25] have proposed the following criteria that could constitute an extragenic safe harbor for DNA integration: A safe harbor should be (i) outside a gene transcription unit; (ii) located >50 kilobases (kb) from the 5′ end of any gene; (iii) located >300 kb from cancer-related genes; (iv) located >300 kb from any identified microRNA; and (v) outside ultra-conserved regions and long noncoding RNAs. In studies of lentiviral vector integrations in transduced induced pluripotent stem cells, analysis of over 5,000 integration sites revealed that ~17% of integrations occurred in safe harbors. The vectors that integrated into these safe harbors were able to express therapeutic levels of β-globin from their transgene without perturbing endogenous gene expression.[24]

Several candidate genomic safe-harbor sites (GSHs) have been explored, including AAVS1, CCR5, and the ROSA26 locus. Although there are clinical data for CCR5 knockout in T cells and other data showing the safety of integration into AAVS1 in human cultured T cells, these sites have not been validated as universal GSHs.[25] In addition, it is not known whether the gene-rich loci of these sites, including some oncogenes, will limit their use when targeting other cell lines.

Of course, much remains to be learned about sites that are identified as being GSHs. Non-protein-coding sequences may be more prevalent than currently known, and some data suggest that there may be low levels of transcription in intragenic sites. Validation of such sites will require measuring the effect of the integrated transgene on neighboring gene expression. This can be more easily accomplished in cell types that can be cloned such as pluripotent stem cells or T lymphocytes, and may not be feasible in cells such as neural cells or hematopoietic stem cells. Moreover, true GSHs would have to tolerate (i.e., without unintended transformation) the integration of a number of different elements, including promoters, enhancers, and chromatin determinants. Finally, validation of the safety of integration at these safe harbors should be done in animals; however, there are challenges in developing appropriate animal models.[25]

Identifying and evaluating the impact of off-target activity

Although innovative designs and exploration of safe harbors are certainly important strategies, any clinical development strategy will have to include identification and evaluation of potential off-target sites. A complete catalog of off-target sites might be accomplished using whole-genome sequencing to look for evidence of indels and translocations. However, this approach would also be very costly, especially for less frequent events, and the sequencing itself has an error rate.[26] For example, if the off-target cleavage occurs at a frequency of 0.1% per genome, at least 1,000 genomes may have to be sequenced to capture such a low-frequency event, adding a significant cost to the analysis. Furthermore, as DSBs and repair can occur in cells as a result of culture conditions alone (even in the absence of exogenous nucleases), there is the challenge of distinguishing the actions of nucleases from naturally occurring background DNA breaks and the spontaneous formation of small indels. In addition, with ongoing deep-sequencing projects, it is now recognized that any individual's genome can contain up to 750,000 unique indels.[27] As a result of these limitations, many groups have used a focused approach to base prediction of potential off-target sites sequence similarity to the on-target site followed by experimental confirmation to validate those predictions. However, the significant disadvantage of such a focused approach is that it will potentially miss other potential off-target sites that could have clinical significance.

Bioinformatics tools are being developed to predict off-target sites. A

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number of web-based tools have been developed, such as Predicted Report of Genome-wide Nuclease Off-Target Sites (PROGNOS, http://baolab.bme.gatech.edu/Research/BioinformaticTools/prognos.html). PROGNOS can provide a report of potential genome-wide nuclease target sites for ZFNs and TALENs. Once a particular target site is identified, the program can provide a rank list of potential off-target sites. These tools are just being developed, and their validation will require more data on actual off-target sites from specific constructs. However, once validated they have the potential to offer a roadmap to search for off-target sites.

In evaluating the nature of off-target sites, the problem is determining what degree of similarity the sequence must possess to lead potentially to binding and cleavage. For example, for the CRISPR/Cas9 system, the 20-bp binding sequence can tolerate mismatches between the gRNA and its complementary target DNA sequence resulting in binding and nuclease action. The degree to which the CRISPR/Cas9 system will bind to these mismatched sequences may depend on the number, location, and nature of mismatches,[21] but larger data sets are needed to discern whether predictive rules can be derived. Binding might occur even if there is an extra DNA base pair (sometimes referred to as a DNA bulge) or an extra RNA nucleotide (an RNA bulge). Based on the analysis of how specific CRISPR gRNAs could still bind with such mismatches together with DNA or RNA bulges, Gang Bao and his colleagues developed a new tool called CRISPR Off-target Sites with Mismatches, Insertions and Deletions (COSMID).[28] This tool is now being further validated using published data on CRISPR off-target sites to evaluate the accuracy of the predictions.

While these tools are elegant and provide useful data, they are just a starting point, as experimental data are needed to validate whether the identified targets are real. As noted by Frederic Bushman, with any target there will be a significant number of potential binding sites, some favored and others less so. While some nonfavored sites appear to be almost as good a match as the intended target, for others small alterations in a similar sequence would prevent binding, e.g., a thymine methylation that leads to a 1,000-fold diminution in affinity. The future for these tools may be to incorporate biochemical data that will allow for some ranking of the most likely off-target sites. With further improvements, these tools will be useful to allow ranking of off-target sites; however, the frequency at which indels occur at these sites will be determined by the specific cell type being manipulated (because different cell types have different mutagenic properties) and the duration and level of nuclease expression.

Unbiased analysis of genome breaks

In addition to identifying potential binding sites for the nucleases, it is important to understand whether DSBs are occurring and what the implications of those breaks are. The field refers to these approaches as "unbiased," as they are attempting to measure the off-target DSBs.

Although the first step in the action of these nucleases is to bind to the recognition sequence, the nuclease must then cut the DNA, and it is the DSB that is the key off-target activity of interest. Several biochemical approaches are being developed not only to look at the potential binding sites but also to see whether there is actual DNA cleavage. Dr Liu and his colleagues examined whether there were actual DNA breaks by ZFNs at off-target sites using an *in vitro* method that combined libraries of potential off-target binding sites and deep sequencing to look for evidence of actual cleavage at those sites.[29] Another method was used to determine the nature of off-target sequences cleaved by two ZFNs (CCR5 224 and VF2468) currently in clinical trials. They created a series (10^{12} total) of mutated half-sites and determined which of those mutations were recognized and cleaved and at what frequency by the two functional ZFNs. Using PCR and deep sequencing, the authors were able to identify specific nucleotide changes that could lead to off-target cleavage by both ZFNs. They experimentally showed that many off-target sequences were present and identified in K562 cells grown in tissue culture.[29]

Drawing from work with integrating viral vectors, Christof von Kalle has developed an assay using integrase-defective lentiviral vectors (IDLVs) to identify off-target breaks. IDLVs—like any other extrachromosomal DNA—occasionally get trapped in a DSB during NHEJ repair, thereby stably marking these otherwise transient and undetectable events. IDLV integration sites in cells treated with ZFNs targeting the human genes *CCR5* and *IL2RG* have been analyzed by linear amplification–mediated (LAM) PCR. A clustering of IDLV integration sites was detected at the ZFNs on-target site indicative of ZFN activity. However, a few other genomic positions show such clustering of IDLVs indicative of off-target activity at those loci. Molecular analyses confirmed that off-target activity occurred at genomic positions bearing homology to the ZFNs target site. With the detection of ZFNs off-target binding sites, one could then measure the frequency of off-target cleavage at a specific off-target site by deep sequencing to identify the exact nucleotide positions within the ZFNs target sequence that tolerate nonspecific sequence recognition, thereby contributing to off-target activity. Interestingly, the presence of a highly homologous sequence did not reliably predict off-target activity, indicating that additional unknown cellular factors also influence target site recognition. Using a similar technique, the specificity of TALENs targeting the human COL7A1 gene was analyzed. Only three off-target positions could be detected by the described IDLV capture approach. Thus, experimental determination of the off-target activity for each designer nuclease may be required. These experiments represent an approach to move toward genome-wide determination of designer nuclease–associated off-target activity but also demonstrate that ZFNs and TALENs can modify the host genome with an extraordinarily high selectivity.

Although this method is promising, the lower limit of sensitivity remains to be defined and the use of a viral vector has the potential to introduce some bias into this analysis. The question then becomes whether it is important from a clinical perspective to capture all DSBs or just those that happen at a particular

frequency. It was noted that as cells are cultured over several days, there is always the potential for DSBs, thus some background level is already tolerated.

Recently, Dr Joung's lab described an unbiased, sensitive, and genome-wide approach for identifying DSBs induced by CRISPR/Cas9 nucleases. This method, known as genome-wide unbiased identification of DSBs enabled by sequencing (GUIDE-seq), relies on capture of short double-stranded oligodeoxynucleotides (dsODNs) into CRISPR/Cas9-induced DSBs in cultured human cells.[19] Fragments of genomic DNA harboring the dsODN can be selectively amplified, sequenced, and mapped back to the genome to precisely identify DSBs to the nucleotide level. GUIDE-seq profiles of 10 different gRNAs show that the number of off-target DSBs can vary widely from more than 150 to none detectable. This method provides the first genome-wide method for defining DSBs induced by CRISPR/Cas9 nucleases and should provide an important tool for preclinical evaluation of the specificities of these reagents. This method seems to be more sensitive than the prior viral based methods of capturing off-target DSBs, because significantly more oligonucleotide can be introduced into the cell. Since this method is sensitive and does not require any specialized expertise in viral production—although it does demand significant bioinformatic expertise to sort out true off-target DSBs from noise—it is likely to become an important approach to assessing the specificity of any given nuclease or its variants.

Another assay that has been used to detect DSBs utilizes the gamma-H2AX histone protein. A DSB occurring in this region leads to phosphorylation of H2AX and the formation of a gamma-H2AX protein. An antibody to gamma-H2AX is available and therefore can be used to quantify DSBs that result from off-target nuclease activity.[30] It is important to note that there is a background level of gamma-H2AX due to spontaneous DSBs. In K562 cells, Matthew Porteus's lab evaluated several different constructs using this method, bringing about the binding of (i) a pair of ZFNs to two different target sites, (ii) a pair of TALENs to three different sites, and (iii) a CRISPR/Cas9 to a single target site. Different doses of the nucleases were used. In this experiment, the higher dose of ZFNs resulted in a significant gamma-H2AX signal with a dose response, which was also observed with the use of TALENs, but with the CRISPR/Cas9 there was no increase in gamma-H2AX. The CRISPR was active at the target site, raising the question as to whether there is something different about the CRISPR/Cas9 break that is not well understood and does not result in the formation of gamma-H2AX.

It is important to note that many of these studies have been done in cell lines derived from tumors. Therefore, validation will still have to be performed in cell lines that are the clinical target. Another complexity is that genetic polymorphisms may make it more difficult to predict the potential off-target effects for any individual in human applications. The ideal would be to have an assay that could assess the potential for off-target sites not only in a particular cell type but perhaps even in an individual's cells (e.g., the GUIDE-seq approach could be performed in the patient's cells). While this may be the ideal, it is not necessarily a prerequisite for proceeding to clinical studies if the cumulative evidence in a relevant model provides a favorable risk–benefit ratio.

Chromosomal rearrangements: will they occur and should we be concerned?

What are the potential effects of these off-target breaks? Dr Bushman and David Roth presented their research on chromosomal rearrangements, which may lead to more severe toxicities than those caused by indels. Dr Bushman described work by Kathy High, president and chief scientific officer of Spark Therapeutics, using adeno-associated virus (AAV) vectors to deliver ZFNs to correct factor IX in a hemophilia mouse model. In this experiment, the ZFN nuclease was used to cleave the factor IX target site, and then a second AAV vector delivered the wild-type exons to recombine into the targeted break made by the ZFNs.[31] The AAV provided a marker from which to sequence out into the flanking DNA to determine where the breaks occurred, much like the work of Dr von Kalle with the IDLVs. Southern blot analysis indicated that 2% of the factor IX alleles (2% of the haploid genomes) were modified by homologous recombination. However, qPCR detected that 40% of the haploid genomes had an integrated AAV vector, much more than those that had undergone homologous recombination. This suggested that there may be up to 20 times as many off-targets as compared to on-target sites. By using deep sequencing the authors were able to examine the NHEJ sites and determined that approximately 3% of the sites were on-target and the remaining were off-target.[31] Thus, of the 40% of haploid genomes with AAV vector integration, only 1.2% had NHEJ-integrated vectors at the knockin site. In addition, they examined the number of AAV integrations seen in cells that were transduced with an AAV vector expressing luciferase compared to the cells transduced with the ZFN-AAV vector and detected fewer AAV sites in the ZFN-AAV transduced cells, suggesting that the ZFN-AAV may have some cellular toxicity compared to the AAV-luciferase.

Another set of studies examined chromosomal translocations generated with exposure to different nucleases, not just ZFNs. In the event of simultaneous on-target and off-target breaks, these may re-ligate, creating the potential for translocations, deletions, or inversions. Dr Bushman described a series of experiments in which cells were exposed to ZFNs, TALENs, or CRISPR that targeted different genes—those encoding CCR5, VEGF, and β-globin. Each of these nucleases was shown to have the expected on-target effect. However, they also saw high levels of translocations in all of the nuclease-exposed cells and fewer in controls transduced with a GFP-expressing vector. It remains to be determined to what degree these translocations would have clinically significant effects.

To further examine the significance of translocations, Dr Roth has focused on well-known naturally occurring nucleases, RAG1 and RAG2, that mediate recombination of the gene segments in T cells to create a diverse repertoire of TCRs, known as V(D)J recombination. The RAG nuclease has considerable specificity, cleaving only certain sites, known as recombination signal sites (RSSs).[32] Rejoining of the DSB occurs by NHEJ. The system is not perfect,

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and occasionally an authentic cleavage site is joined to closely related off-target sites, known as cryptic RSSs. More recently, NEHJ has been detected between two off-target events (i.e., breaks made in two different cryptic RSSs), resulting in leukemia.

Dr Roth described an examination of the chromosomal abnormalities in a mouse that had a mutant p53 gene, making it prone to tumors, but expressing wild-type RAG. In addition to chromosomal changes that would be expected in the mutant p53 background, some tumors seemed to arise from RAG-mediated translocations, in that they were located near known cryptic RSSs. A number of these breaks near these cryptic RSSs resulted in deletions that activated oncogenes. Two recent articles showed that RAG nucleases could be the driver of leukemia through off-target cuts resulting in recombination and deletions.[33,34] The relevance of this finding to work with other nucleases is that even with a well-conserved, specific nuclease, there is the potential for off-target activity to have significant biological consequences.

Engineered nucleases with arguably lower specificity might likewise lead to unpredicted and significant genomic rearrangements. In order to determine whether this might occur with TALENs, Dr Roth used TALENs designed to correct the β-globin locus. Genome-wide sequencing revealed deletions that were of equivalent size to those seen in some of the RAG-induced tumors. A number of translocations were also found in chromosome 11 where the hemoglobin gene is located. Although this was a limited analysis, the discovery that off-target activity by RAG can lead to tumors and the discovery of similar types of translocations with a β-globin-specific TALEN indicate that deletions resulting from off-target genome breaks may be biologically significant.

In recent work, Dr Joung and colleagues reported that translocations can occur between CRISPR/Cas9-induced on-target and off-target DSBs identified by GUIDE-seq[19] Interestingly, these investigators also observed that translocations could occur between nuclease-induced DSBs and nuclease-independent DSB hotspots also identified by GUIDE-seq. This latter observation suggests that it is important to consider not only the breaks caused by exogenous nucleases but also how these DSBs interact with any fragile sites that also exist in the cell type of interest. The creation of translocations between the intended nuclease target and random DSBs on other chromosomes has also been confirmed using a LAM-PCR high-throughput, genome-wide, translocation sequencing approach.[35] These two methods of detecting engineered nuclease-induced chromosomal translocations are likely to become an important new approach to assessing the safety of a genome editing process and raises the concern that targeting a site that is associated with cancer translocations might have an increased safety risk.

Functional toxicity assays: genotoxicity, and cytotoxicity

Identifying off-target sites and potential chromosomal rearrangements is critical to assess the safety of new constructs. For developing clinical applications, the question was raised as to which studies would be most useful for evaluating clinical toxicity. Studies that may be able to identify all genome alterations, including those that have not been correlated with any cellular toxicity or clinical adverse effects, may provide important scientific knowledge but may not necessarily be most relevant for preclinical development. Although these basic science studies may be at the extreme edge of sensitivity, functional studies are needed to validate potential toxicity for preclinical development.

Dr Cathomen noted that conceptually for a given concentration of a nuclease, you will have on-target and off-target activity. The ideal concentration will be one in which you have high on-target activity but low off-target activity (**Figure 3**, bottom). If a nuclease has low specificity, then the two curves are closer to one another and the ability to reach an effective dose without toxicity is limited (**Figure 3**, top). There are two main toxicity concerns specific to genome editing technologies: cytotoxicity and genotoxicity. A number of approaches are being developed to measure cytotoxicity. In one assay, GFP^+ cells are used to track cell viability. Cells are co-transfected with a GFP expression plasmid and a nuclease expression plasmid (not all cells are transfected). If the nuclease activity results in toxicity, there will be reduced replication or death of the GFP^+ cells, leading to a decline in the relative percentage of GFP^+ cells over time. This provides a quantitative assessment of toxicity. When this assay was used with selected ZFNs and TALENs, cell viability was inversely related to the dose, suggesting that nuclease concentration may affect viability. To examine the effect of nuclease concentration on cell viability in primary stem cells, ZFNs specific for enhanced GFP (eGFP) have been employed in keratinocyte stem cells derived from an eGFP-transgenic neonatal mouse. ZFN on-target activity should eliminate GFP activity. At different ZFN doses, high levels of on-target activity were seen, although as reported by others, cytotoxicity did increase with a higher dose of ZFNs.[36] Under optimal conditions, the stem cell potential of the keratinocyte stem cells was not altered by the ZFNs.

GFP tagging can also be used to detect clonal dominance, which is often, but not always, a precursor of genotoxicity. Cells were marked with a "barcode" (a short nucleotide sequence that provides a unique identifier for each cell, either by lentiviral transduction or by ZFN-mediated targeted integration). By using deep sequencing, the clonal dynamics of the population can be studied over time. When the barcodes were introduced semi-randomly by lentiviral vector integration, there was evidence

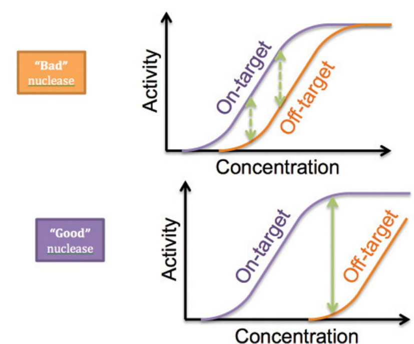

Figure 3. Plots showing the relationship between concentration of a given nuclease and on-target vs. off target activity. A "good" nuclease should exhibit high specificity and affinity for its cognate binding site such that the mass action equilibrium will not shift in favor of off-target sites with small increases of nuclease concentrations. "Bad" nucleases with low specificity are prone to bind more off-target sites with small changes in nuclease concentration and thus limit the ability to reach an effective non-toxic dose. Courtesy of Toni Cathomen.

of spontaneous clonal changes in the population but none that could be reproducibly attributed to the lentiviral insertion. In contrast, the population of cells marked by ZFN-mediated recombination at a single safe harbor showed greater clonal skewing, with the clonal dominance occurring reproducibly from the same clones.[37] It is unclear why the integration of the ZFNs led to additional clonal growth, but the use of K562 cells and high doses of ZFNs may have affected these results. Furthermore, although this assay suggests that changes in clonal dynamics induced by engineered nucleases can be detected, the clinical relevance has not been validated.

Dr Cathomen's group examined the ratio of on-target to off-target events using ZFNs and TALENs specific for CCR5 and AAVS1. When assaying the genotoxicity of ZFN and TALEN pairs targeting CCR5, they initially focused on the known primary off-target site, CCR2. Overexpression of a highly specific TALEN pair did not result in detectable chromosomal rearrangements; however, these were detected following use of the CCR5-specific ZFN. The ratio of CCR5/CCR2 specificity for the different nucleases was determined. The CCR5-specific TALENs had a specificity ratio of either 130:1 or 7:1, depending upon the target sequence, whereas for the CCR5-specific ZFNs, it was 3:1. This difference in specificity paralleled the detection of large chromosomal deletions or inversions in the area of the CCR5/CCR2 loci, where a highly specific TALEN pair did not induce chromosomal rearrangements, but CCR5-specific ZFNs and the TALEN pair that had the lower ratio of specificity demonstrated high off-target effects.[38] Using the bioinformatics tools described above (PROGNOS), they found that the predicted ratio of on-target to off-target sites for the ZFN pairs targeting CCR5 and AAVS1 was about 1:2, and the ratio for the CCR5-specific TALENs was 60:1; and for the TALENs targeting AAVS1 the ratio was 27:1. These constructs were then tested at high doses in HEK293 cells, higher than would be done for physiological dosing. About 80% of the cells transfected with TALENs expression vectors survived compared with about half of cells transfected with the ZFN-encoding plasmids.[38] They further examined the effect on the cell cycle. Extensive cleavage at off-target sites would probably result in arrest of the cell cycle until the DSBs are repaired. Using HeLa FUCCI cells that express fluorescently tagged cell cycle indicators, they again compared the ZFNs to the TALENs. Compared with control, the TALENs did not affect the cell cycle while the ZFNs did lead to more cells blocked at the G2 stage; after 3 days, more apoptosis was observed in those cells where the cycling was disrupted.

What can be concluded about these results, especially in light of at least one ZFN against CCR5 moving into the clinic successfully with no evidence of clinical toxicity and several years of follow-up? Dr Cathomen noted that these results cannot be interpreted as ZFNs being generally less specific than TALENs. Rather, it will be important to carefully evaluate the genotoxic potential of every designer nuclease intended to enter clinical trials. Dr Porteus noted that these assays raise interesting questions, but there is no evidence that they have predictive power for clinical outcomes. The majority of these assays have been done in cell lines using delivery strategies and/or doses that would not be used in clinical applications. Dr Cathomen added that the other assays being used to assess genotoxicity—karyotype analysis and array-comparative genomic hybridization—are relatively insensitive. They may best serve as screening and comparison tools between platforms or as refinements to current platforms. If they are to be adapted to provide data regarding functional toxicity for clinical applications, they will have to be conducted in the cell types of interest, with the nuclease delivered as it would be in a clinical trial.

The role of animal models

Although *in vitro* models are important to guide development of new approaches, animal models have always been used to more definitively explore toxicity. In the context of these agents, animal models potentially allow for assessment of the viability and functionality of the modified cells in an environment in which they will compete with unmodified cells. In addition, for applications where the engineered cell is a precursor or stem cell, animal models can support cell differentiation, which may not be possible *in vitro*, and thereby make it possible to more rigorously assess functionality. Furthermore, the cell expansion that occurs *in vivo* can amplify genotoxic events and allow the outgrowth of tumorigenic cells resulting from a very rare event. However, the use of animal models to assess DNA sequence–specific reagents such as nucleases can be complicated by differences between the human and animal target sequences, so that the reagents used may not recognize the analogous nonhuman sequences. As one approach to circumvent this problem, the gene therapy field has adopted the use of immunodeficient mice that can support the engraftment and differentiation of human hematopoietic systems, and these have been used to assess genotoxicity of integrating vectors in these cell tissues.[39] Such an approach may now also be used to assess endonuclease-based approaches in hematopoietic stem cells.

Paula Cannon reviewed some key elements of these types of experiments. Because the recipient mouse strains are immunodeficient, if tumors do arise in such mice, investigators must characterize these tumors and evaluate whether they are of human origin. If tumors are of human origin, then it will be necessary to further evaluate their clonality with respect to a nuclease modification signature at any of the on- or off-target sites. However, clonality observed in a nuclease-modified cell does not necessarily equal causality and may instead be an innocent label that merely reflects the tumor's clonal origin.

While the models developed for evaluating mutagenic potential for integrating vectors can be adopted for nucleases, at least for nuclease-modified hematopoietic stem cells or T cells,[40] an important caveat is that these models have not been able to recapitulate the clinical tumors seen in the human trials with integrating viral vectors. Although insertions near oncogenes can be documented, the mouse models have not demonstrated the vector-driven leukemia seen in some subjects. The lack of toxicity in animal models will be an important safety check, as one would not want to proceed in the face of animal toxicity. However, the chal-

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lenges in developing appropriate, efficient animal models to evaluate genotoxicity and in particular oncogenicity for integrating vectors have been documented,[41] and similar challenges may arise as these models are used to evaluate genome editing technologies.

Bringing it all together: moving into the clinic

At the time of this meeting, only ZFNs have advanced to clinical trials. The first successful clinical application for genome editing has been seen with a ZFN targeting CCR5, a receptor expressed on T cells that allows HIV to enter.[2] Phil Gregory and Dale Ando, Sangamo BioSciences, reviewed their path to the clinic. Development of this product began in 2003 when many of the assays reviewed during the meeting were not yet available. Dr Gregory noted that the path taken for these products is in some ways the same for any pharmaceutical development, including generating therapeutic reagents that are maximized for potency/specificity and working within established regulatory frameworks to characterize the safety of the product. For genome editing, an additional challenge that is unique to this class of "drugs" is the need to define specificity in addition to classical toxicology assessment. Also, identification of off-target sites is complicated by the lack of a clear footprint in the genome, unlike those of integrating vectors, which could be easily detected.

One of the first steps with this platform development was to maximize the specificity of the ZFNs. As discussed above, the ZFN modules each recognize three base pairs. These modules can be combined so that the interface is highly specific. In addition, the linkers between the modules and the links between the Fok1 nuclease and the modules can be altered to maximize engagement of the preferred sequence. Moreover, the Fok1 domains can be engineered to require heterodimer binding. Because of these variables, up to 10^5 ZFN dimers can be generated for a particular exon region. The selection of a candidate to move forward into the clinic required the use of bioinformatics and selection technologies such as phage display[42] to identify the product that is maximized for specificity and activity.

The ZFN-CCR5 used in clinical trials developed by Sangamo, known as SB-728-T, was delivered by an adenoviral vector into mature T cells. To determine the impact of this nuclease on the T cells, Sangamo undertook a series of assays in which the phenotype and the growth kinetics of the modified T cells were compared to those of unmodified T cells. Cytokine release was also determined to be the same in modified versus unmodified T cells, and there was no skewing of the diversity of the TCR variable domain. Finally, it was determined that the CCR5-modified T cells are stable in the population in the absence of HIV infection, and HIV infection leads to enrichment of the CCR5-modified T cells.

In addition to these experiments, it was important to analyze the fidelity of the genome editing. The first step was to focus on molecular assays that could identify primary off-target action, i.e., DSBs. Although bioinformatics tools were a starting point, an unbiased approach was also needed. One can deep-sequence sites that are the closest matches to the consensus site for the ZFNs binding sites, as identified using bioinformatics tools. This deep sequencing can detect events as rare as 1:10,000 alleles but is limited by the fact that the initial screen is identifying a relatively small number of sites per sequence. One can also use immunostaining for DSBs, which is unbiased but again limited to about 100 individual nuclei per conditional time point. As discussed above, genome-wide assessment of ZFNs-induced DSBs using IDLV capture and nonrestrictive LAM-PCR is another approach, but it is limited by the sensitivity of IDLV capture at rare DSBs. Nonrestrictive LAM-PCR does not utilize restriction enzymes to cleave the genomic DNA, which may reduce sensitivity to detect some sites, but its use of sonication shearing should increase sensitivity. Finally, karyotyping of cells can reveal genomic rearrangement, but the number of cells analyzed per sample is low. In addition, nonclonal rearrangements are often present in untreated cells, making it important to also determine the background incidence.

In the development of the CCR5 product, Sangamo identified 15 potential off-target sites and then used deep sequencing (454 sequencing) to look for DSBs at the identified sites. This allowed for identification of one off-target site in 1:20,000 alleles, and the identification of one other site, CCR2, that had about a 4% frequency of DSBs compared with ~35% at the CCR5 target. Immunostaining for DSBs was also done using p53-binding protein1 (53BP1), which is recruited to sites of DSBs early in their repair and is required for NHEJ.[43] Importantly, there is a background rate of positive sites reflecting the physiological incidence of DSBs. Transduction of the cells using the adenoviral vector carrying the CCR5-specific ZFNs resulted in an increase in DSBs of 1.4–1.6% compared with 4.1% in the presence of a chemotherapy agent etoposide. Because CCR2 is in close proximity to CCR5, it was not possible to visualize two independent 53BP1 foci by staining.[43]

Following these molecular assays, a series of *in vitro* and *in vivo* assays were conducted to establish safety and in particular, the absence of oncogenic potential. *In vitro* oncogenicity assays were based on the experience in previous gene therapy T-cell product characterizations. In addition, because the cancer chemotherapy cytotoxic agents have the greatest genotoxic and carcinogenic potential, standard *in vitro* studies for preclinical evaluations of these types of drugs were also used. The ability of a primary T cell to grow without cytokines and cell signaling is a feature of carcinogenic transformation. Culturing of SB-728-T cells without cytokine support was performed for weeks and demonstrated that normal cell death occurred. The classic biological cell transformation assay is anchorage-independent growth of fibroblasts and is a stringent test of carcinogenesis. These fibroblasts are also amenable to gene transfer by adenovirus, so delivery of the ZFNs into these cells can be achieved. The US Food and Drug Administration (FDA) asked Sangamo to use as high a multiplicity of infection as possible for these tests. Other tests evaluated but not chosen to evaluate for oncogenicity were the Ames test, Mouse micronucleus test, and mouse lymphoma TK gene mutation assay.

SB-728-T *in vivo* safety studies relied on the fact that human T cells could be maintained in immunodeficient NOG mice. After discussion with the FDA, Sangamo tested a full human dose of modified T cells (using T cells from three different donors), allowed the modified human T cells to live and expand for months in the NOG model, and tested this against T cells modified using a maximal multiplicity of infection in order to recreate a "worst case" scenario. It took some time to develop a model with

human T-cell xeno-GVHD, defining that 2 months was a maximal time for proliferation of cells before animals died of GVHD, and defining a dose and donors that gave reliable GVHD in the NOG mice. Three studies were performed (one for each donor), with a duration of 2 months, and then animals were euthanized; all organs were evaluated by histology for neoplasms, immunostaining to detect human cells, and PCR for detection of ZFNs CCR5 modification at on-target and off-target sites. Histology was consistent with GVHD in liver, lung, gut, and spleen, showing intense inflammatory infiltration. Human DNA was present in all animals, and there was equivalent engraftment of modified and control cells. No CCR5-related CD4 T-cell neoplasms were found.

These studies led to the first genome editing phase I trial conducted by Pablo Tebas and Carl June at the University of Pennsylvania.[2] In that study, administration of CCR5-modified T cells was safe, marked increases in total CD4 T cells were observed, and the modified T cells trafficked normally to the gut. One subject achieved controlled HIV viral load below levels of detection after an antiviral treatment interruption of 12 weeks. The ZFNs-modified T cells persisted at a level >50 modified CD4 T cells/μl for 252 weeks.

Putting it all together for future clinical applications

The meeting reviewed a number of assays that are being developed but are yet to be validated. The question remains how to best integrate these assays into the preclinical development strategy. A goal for the field may be to build assays prospectively for a particular outcome, defining the sensitivity and cutoff values in advance. It was noted that although the meeting proposed to focus on "establishing preclinical toxicology standards," the main focus was on detecting and evaluating off-target effects. It was acknowledged that one way to evaluate these technologies is using an unbiased approach such as whole-genome sequencing to understand where these nucleases act in the genome, but the cost and potentially low sensitivity makes this approach impractical for preclinical development. Instead, the goal has been to find predictive tools that allow for a more focused evaluation of the most likely off-target sites with significant activity.

One question is whether it is necessary to detect every potential indel. Is there a level below which one could be confident that the frequency would not lead to a clinically significant lesion? Does a focus on the most frequent off-target sites, perhaps with a particular focus on translocations, sufficiently help ensure safety? It is unlikely that the nature of the repair product can reliably predict function, as not all deletions are benign; moreover, although translocations are frequent in tumors, there may be many translocations that do not lead to transformation events. Therefore, it may not be necessary to identify all off-target activity but rather to develop assays that reliably identify transformation of cells. In addition, as the specificity of these nucleases improves, the frequency of off-target DSBs may decrease to a level that may reasonably allow the analyses to focus on the most prevalent of the off-target sites.

Assay development may be an essential milestone in the development pathway before a commitment is made to a particular engineered nuclease. One could begin with using biochemistry/bioinformatics/molecular assays to evaluate candidate nucleases and their potential for off-target and cellular toxicity. These results may then be used to optimize the products. Once the optimal specificity has been engineered and any off-target sites identified, there will be a need for more classical measurements of toxicity and genotoxicity, both *in vitro* and *in vivo*. Importantly, these assays may have to be adapted to focus not only on the nuclease but also on the proposed method of vector transduction and the cell context.

On a more practical level, this is a rapidly developing field, with new ways to assess the activity of these constructs emerging at an equally fast pace. Ultimately decisions will need to be made regarding which assays are scientifically valid and which are needed to satisfy the legal-regulatory framework. There are a number of assays that may provide some data on safety, but each has limitations in terms of sensitivity. When assessing the impact of rare events, the question is often how many negative readouts are needed to provide confidence that one can proceed into the clinic. This risk–benefit calculus must also take into account the proposed disease target. For example, if these approaches will offer alternatives to allogeneic transplants—the standard of care—then the risk of an off-target event that might have an unforeseen clinical outcome should be weighed against the known 20–30% risk of GVHD and a 5–10% risk of death.

Another safety feature may be the choice of initial cells to target with these tools. Sangamo Biosciences conducted their initial ZFNs clinical trial in a terminally differentiated T cell. New trials are exploring the same construct in stem cells. If one looks at the experience in gene therapy, the same type of retroviral vector that caused leukemia when used in hematopoietic stem cells in trials for X-linked severe combined immunodeficiency, chronic granulomatous disease, and Wiskott-Aldrich syndrome, has not led to leukemia in other protocols that have used terminally differentiated T cells, even though the ability to transform T cells in a preclinical setting has been demonstrated.[44] Monitoring the behavior of these nucleases in differentiated cells, and looking for normal activity and differentiation, provides some data that these nucleases are not disrupting cellular activity through action at other loci. However, the counterargument to this is that safety in the differentiated cell does not guarantee safety in the stem cell.

Several new investigators in this field come from the gene therapy field, and in particular the area that uses integrating vectors to accomplish long-term gene correction. The genome editing field draws on the experience of those studying integrating vectors. However, there are limits in the ability to extrapolate from the experience with integrating vectors and their assays. For example, in the field of gene therapy, one group looked at integration sites for the same vector in T cells vs. stem cells and found different integration patterns. However, when the ZFN for CCR5 was examined in T cells and hematopoietic stem cells, there were no differences in the off-target sites for the CCR5 in T or $CD34^+$ cells, underscoring that while vector integration is largely random, the action of these products is more directed, including any off-target events.

It is as yet unclear if the preclinical pathway for these technologies will differ depending upon whether the goal is gene disruption, correction, or insertion. Certainly there would be additional challenges when

moving beyond gene disruption—which has been the focus of clinical applications to date—to gene replacement. Gene correction faces the challenge that DSBs could also be repaired by NHEJ; therefore, if this approach is to be safe, site selection must be such that disruption would not lead to adverse effects.

It remains to be determined whether genome editing to achieve gene addition will be safer compared to gene therapy approaches using randomly integrating vectors. Although there is considerable experience with gene delivery via integrating vectors, efficient gene delivery into a predetermined site using nuclease technology and homologous recombination is still being developed. The therapy that will prevail may not be the most elegant but must be proven to be safe, effective, and easy to implement at multiple manufacturing sites, and provide an advantage over treatments or approaches that currently exist. Targeting expression from a specific site, even if well characterized, may not provide more efficacious levels of expression than the expression from multiple random sites achieved with integrating vectors.

There remains a gap in our current understanding of certain aspects of these technologies that may be important in evaluating clinical applications. For example, in developing new TALENs and CRISPRs, when a reagent does not work at the expected target, it has been relatively easy to adjust the sequence and manufacture a new one rather than exploring why the agent did not work.

An ultimate goal would be to establish regulatory pathways requiring well-understood, standard assays, so that one could sequence the genome of a patient or infant, identify a target, make sequence-specific reagents, and (because of the similarity to the approaches that have been previously successful in the clinic) develop a personalized reagent that is ready for clinical use in 2–3 months. The development of such a well-defined validated process may be the ultimate path for precision medicine.

ACKNOWLEDGMENTS

The authors thank all the participants for their contributions and Dale Ando for assistance in summarizing his presentation. The views expressed in this report are those of the individual authors and do not represent the views of the NIH.

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