Targeting safety in the clinic for precise genome editing using CRISPR: a genotoxicologist's perspective

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The genome editing revolution

Although the concept of gene therapy arose decades ago, it has been slow to realize its full potential. This has been, in part, due to concerns associated with early clinical trials and difficulties with safe and accurate targeting [1]. However, with the emergence of precise genome editing (PGE), we are on the brink of a gene therapy revolution. Genome editing with protein-based guides (zinc fingers and transcription activator-like effector nucleases) have already been utilized as therapeutic modalities. However, it is the simplicity of the RNA-guided CRISPR technology that is now leading the field in the development of PGE. CRISPR guide RNA (gRNA) associated with an endonuclease (e.g., Cas9), is a prokaryotic defense mechanism that targets and cuts invading viral DNA. Doudna and Charpentier's harnessing of this and the development of single gRNAs and associated endonucleases to edit human DNA has opened a biotechnology 'Pandora's tool box' of possibilities, not least to edit and 'fix' DNA defects [2]. However, alongside excitement for a new frontier of experimental medicine comes the realization that we are entering uncharted ethical and safety territory. From 1–3 December 2015, the great of the genome editing community met in Washington (DC, USA) at the International Summit on Human Gene Editing, to discuss ethical and governance issues [3]. Their main concerns were around the impli-

cations of editing the human germline, but with the consideration that the technology holds “many promising and valuable clinical applications directed at altering genetic sequences in somatic cells.” While this was a green light for investigations into somatic cell DNA disorders, there are theoretical safety concerns that need to be addressed before genome editing can become a mainstay therapy. The main safety consideration is around how precise PGE really is, in other words, is the intended DNA target the only edited target? Unsurprisingly, the concept of treating humans with nucleases that target DNA and induce strand breakage is an emerging hot topic in pharmaceutical genetic toxicology. We are used to assessing and understanding the safety profile of small molecule drugs, where direct DNA interactions can halt development in all but the most life threatening of conditions. For small molecules, the 'one-hit' DNA damage theory still holds sway, in other words, there is no safe therapeutic level of a DNA reactive small molecule mutagen, as a single mutation at a relevant gene target (e.g., oncogene) can lead to cancer. While there is much debate around the scientific credence for this consideration, it is still a major regulatory concern. Hence how do we begin to develop and safety assess CRISPR therapies where, if taken to the extreme, a single off-target event could be interpreted (or perhaps misinterpreted) to be an unacceptable risk?

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Improving DNA target specificity

To address off-target concerns, there is now an enormous amount of research activity into developing proteins and gRNA's with increased specificity, including:

- Improved bioinformatics to predict off-target binding – it is known how many base mismatches can be tolerated (surprisingly over five) and which parts of the gRNA sequence are required to be conserved (generally mismatches at the 5' end are better tolerated) and there is beginning to be an understanding of how chromatin may affect gRNA binding [4]. Increasing the understanding of these dynamics will help in the design of more specific gRNAs;
- Alterations to the RNA guide – truncated gRNA sequences (i.e., fewer than 20 bases) and changes to the protospacer adjacent motif sequence (the guide 'start' sequence) from nucleotide guanine–guanine (NGG) to a rarer sequence have been shown to improve selectivity [5,6];
- Targeting both DNA strands—with CRISPR–Cas9, targeting a site on one DNA strand is sufficient to elicit a double-strand break. However, specificity has been increased by using guides designed either with only one endonuclease which elicit single DNA nicks (nickases) or associated with the dimer requiring FokI endonuclease. In both cases separate and more target specific guides to each strand are required for effective double-strand cleavage [7,8];
- Use of alternative endonuclease proteins – the most commonly used endonuclease has been *Streptococcus pyogenes* Cas9, but CRISPR-associated endonucleases are common in many prokaryotes and many variants exist, some of which are demonstrating improved specificity and it is highly likely that more target-specific variants are yet to be discovered [9].

Learning from the past

CRISPR technology is still in its infancy, with barely three short years of development, so DNA target specificity can only improve. However, toxicologists should not be complacent; improved tools to demonstrate the specificity of CRISPR guide therapies are still required. Furthermore, safety lessons from new modality therapies of the past should be remembered, for example, since the 1990's anti-sense oligonucleotide therapies have been dogged with unfounded concern around potential mutagenesis from higher order binding (e.g., DNA triplex formation) [10] and

the possible genotoxicity of modified nucleotides [11]. This anxiety was not based on bad science but the lack of an understanding of the translation of rare *in vitro* events to the clinic or the inevitable difficulty with proving a negative, plus the conservatism associated with genotoxic risk assessment. Hence, it has taken two decades for these concerns to be shown to be irrelevant [12]. If PGE is going to develop as a life-changing therapeutic modality, we should endeavor to ensure the correct science is done now and most importantly the findings are put into an appropriate perspective. There are attempts to do this, a recent *Nature Perspective* from George Church (one of the pioneers of therapeutic CRISPR) calculated that 'in the best case scenario' CRISPR–Cas9 is capable of less than one error in 300 trillion base pairs, given that the human genome is only 3 billion base pairs, that equates to one off-target hit per 100,000 cells and even then it would be extremely unlikely that it would be in a relevant gene [13]. Furthermore, it is estimated that each human cell is subjected to around 20,000 endogenous DNA lesions per day, of which the vast majority are efficiently repaired [14]. Hence is the contribution of off-target CRISPR editing really significant? Unfortunately, Church's calculation is based on limited experimentation in two cell lines [15]. That does not mean that the estimate is false, but it does mean that more work is needed to confirm this level of safety translates to humans.

Measuring off-target effects

In a bizarre way, CRISPR is a victim of its own precision. When zinc finger technologies were initially utilized as potential therapeutics, the depth of sequencing that is currently available was not possible. Furthermore, because CRISPR off-target events are so rare, even currently available sequencing technologies cannot identify them above background error. Therefore, for safety reassurance, there is a need to develop specific unbiased techniques. Advances are already being made, with GUIDE-seq developed in Joung's Harvard laboratories [15] and Digenome-seq developed in Kim's Seoul laboratories [16], as recent examples. It has been demonstrated that computational analysis of the genome does not accurately delineate CRISPR–Cas9 cleavage sites. Furthermore, use of dead Cas9 showed that the relationship between actual target binding and DNA cleavage is complicated, with more sites targeted for binding than are actually cleaved [15]. Accordingly, both GUIDE- and Digenome-seq were developed to measure the number of actual cleaved sites, GUIDE-seq by insertion of a tagging oligonucleotide into the break and digenome-seq by analysis of sequence reads with the same 5' ends

from CRISPR–Cas9 cuts. These technologies are paving the way for an appropriate assessment of CRISPR off-target, but development is still needed to monitor off-targets *in vivo*. For human populations, with individual SNPs, the only complete assurance may be to actually prescreen individual patient recipients. In the future, it could be imagined that a simple *ex vivo* prescreen using patient peripheral lymphocytes and unbiased CRISPR off-target sequencing technologies will be used to demonstrate a particular therapy is safe in the relevant patient, at least in one tissue type. This could be coupled with off-target screening work *in vivo* (possibly using humanized tissue) and *in vitro* with differentiated human-induced pluripotent stem cells to monitor tissue differences (e.g., from chromatin modifications). Furthermore, PGE does have the advantage over small molecule mutagens in as much as it is possible to measure exactly where off-target cuts are made, this is very different from the essentially random DNA targeting of chemical mutagens. Accordingly, as the science matures, it can be assumed that even if PGE induces limited off-target effects, these can be identified and measured at the gene level and the consequences assessed.

PGE clinical precedent

Sangamo Biosciences (Richmond, CA) have successfully reached Phase II clinical trials with their zinc-finger editing of the *CCR5* gene for HIV therapy. This used an *ex vivo* approach, so did not have to pass the

higher safety assessment that would be required for systemic delivery (the holy grail of PGE) but Sangamo's pathway to the clinic did show an innovative approach including use of targeted sequencing analysis, *in vitro* cell transformation (oncogenicity) assays and transposed edited T cells into immune-compromised mice for phenotypic *in vivo* assessment [17]. Sangamo's *ex vivo* zinc finger therapy safety assurance was accepted by regulators.

Conclusion

With the development of any new therapeutic modality, there is a danger that unfounded theoretical safety concerns will slow or restrict progress. If PGE with CRISPR–Cas9 is to fulfill its potential as a life changing therapy dealing with significant unmet medical need, a pragmatic approach is needed by both research scientist and regulators. The goal over the next few years is to provide the relevant and human translatable science that will make this possible.

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