Research Highlight: Toward therapeutic genome editing in post-mitotic retinal cells.

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The repertoire of nuclease-guided genome modifications was recently expanded to include homology-independent targeted integration (HITI) of exogenous DNA by a report published in the December 2016 issue of Nature (Suzuki et al. 2016). The key feature distinguishing HITI from homology-based DNA Knock-in is its applicability in post-mitotic cells of non-regenerative tissues, such as the brain and retina. Suzuki et al. demonstrated the therapeutic utility of HITI through genomic knock-in of an exon previously deleted by a naturally occurring mutation that causes blindness in rats. The potential therapeutic impacts are farther-reaching than the replacement of deleted genomic segments. Targeted integration of therapeutic genes in post-mitotic cells *in vivo* may improve treatments that rely on continued gene augmentation or silencing.

There is tremendous promise for treating inherited diseases using clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) technology. Genome editing strategies utilize cellular enzymes to anneal double strand DNA breaks (DSB) created by nucleases (reviewed in Yanik et al. 2017). A caveat for translational application frequently omitted from discussions is that the expression of DSB repair enzymes that use donor-template homology is dependent on cell cycle progression. Recombinases that facilitate homology-directed repair (HDR) are predominantly expressed during late S/G2 phases, while microhomology-mediated end joining (MMEJ) factors that allow precise integration into target chromosome (PITCh) are expressed in M/early S phases (Nakade et al. 2014). For diseased organs composed of proliferative cells, *in vivo* HDR or MMEJ editing can improve function by repopulating tissues with edited cells if genetic correction confers a survival advantage (reviewed in Cox et al. 2015). However, without inducing expression of HDR or MMEJ DSB repair enzymes, these editing pathways are incompatible for use in *in vivo* therapy in post-mitotic tissues.

In contrast to HDR and MMEJ, DSB repair in non-dividing cells is largely restricted to classical non-homologous end joining (NHEJ), as this pathway is exclusively active at all cell cycle stages including G0/quiescence (Symington and Gautier 2011). CRISPR/Cas-mediated gene knockout is one utility ascribed for NHEJ-based editing as DSB repair is error prone. The random number of nucleotide insertions/deletions (indels) during DNA strand annealing frequently generate frameshift mutations that specify early termination codons, which results in functional gene ablation. As such, CRISPR-KO represents a therapeutic strategy to treat monogenetic dominant disorders if pathogenic alleles can be discriminated from wild-type for ablation (Bakondi et al. 2016).

A second NHEJ-based therapeutic strategy proposed is the removal of intronic splice mutations that incorporate non-coding sequences into mRNA transcripts to form pseudoexons. Targeting Cas cleavage at two sites flanking an intronic splice mutation was shown to excise the mutated region and correct transcript splicing in quiescent retinal photoreceptors *in vivo* (Ruan et al. 2017).

A third NHEJ-based strategy to modify genomes in post-mitotic cells is targeted DNA insertion. The feasibility of blunt-end DNA ligation at sites of DBS was previously shown in cultured human cells (Geisinger et al. 2016, Maresca et al. 2013), zebrafish embryos (Auer et al. 2014), and the

first therapeutic demonstration was provided by Suzuki et al. In the highlighted article, the investigators corrected the mutation in the Royal College of Surgeons (RCS) rat model of retinitis pigmentosa (RP). The genetic defect in RCS rats originated by spontaneous deletion of exon 2 of the mer proto-oncogene tyrosine kinase (*Mertk*) gene that occurred generations earlier (D'Cruz et al. 2000). Joining of exons 1 and 3 shifts the reading frame and truncates Mertk into a non-functioning peptide in quiescent retinal pigment epithelial (RPE) cells. This leads to progressive photoreceptor loss and vision decline and models some forms of RP in patients. Using adeno-associated virus (AAV) for translational delivery to the retina, a HITI vector was used to restore exon 2 to *Mertk* and correct the reading frame. This resulted in full-length Mertk expression and partial vision rescue. However, mutations in the orthologous *MERTK* gene in patients are heterogeneous and not amenable to correction using the strategy designed for RCS rats. While these data suggest feasibility of using HITI to correct analogous deletion mutations, the study rather demonstrates feasibility for phenotypic and functional improvement by genomic knock-in, which suggests benefit to other gene therapies via HITI.

Therapeutic impact may come from utilizing safe harbor locus integration for gene replacement therapy. Gene delivery by lentivirus is experimentally useful as stable genome integration allows constitutive vector expression, but risk of insertional mutagenesis from random integration may prohibit patient use. Conversely, the episomal nature of AAV vectors makes them safe but limits their duration of vector expression (Trapani et al. 2014). Alternatively, AAV delivery of HITI vectors may allow safe integration of therapeutic transcripts for constitutive or controlled expression. This may be especially advantageous for therapies that rely on continued expression of effectors that non-permanently activate or repress endogenous gene transcription (e.g. Perez-Pinera et al. 2013 and Qi et al. 2013) or those that silence mRNA translation (e.g. Liu et al. 2016). Proof of principle for safe integration was shown by knock-in of an AAV-HITI-GFP vector at the Rosa26 locus following intramuscular injection in adult mice (Suzuki et al. 2016)

Additional applications for HITI may come from the observed rates of Knock-in in dividing cells. Comparison of *in vitro* editing efficiencies between HITI, HDR, and PITCh vectors in proliferative human cells (HEK293) revealed that the highest rate of GFP Knock-in was from the HITI vector. Knock-in efficiency in organs containing dividing cells was also highest using the HITI vector. Intravenous delivery of AAV-packaged vectors showed higher targeted knock-in by HITI compared with HDR in heart and liver cells (Suzuki et al. 2016). Thus, the use of HITI may improve or replace some *in vivo* editing strategies that currently utilize HDR or PITCh. Toward this end, Suzuki et al. has addressed the size limitation of vectors for packaging into AAV particles by demonstrating the convergence of HITI effectors delivered in separate AAV vectors to induce DNA knock-in *in vivo*. Although demonstrations of direct translational editing strategies are needed, HITI has expanded the repertoire of possible modifications in post-mitotic cells *in vivo*, and thus the number of disorders treatable by therapeutic genome editing.

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