

EDITORIAL



In vivo genome editing as a therapeutic strategy for the treatment of inherited retinal dystrophies: how close are we to realization?

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While the expectations for genome editing to treat inherited retinal dystrophies are high, fundamental questions still remain unanswered, making this approach highly risky.

Specific advantages of the eye as target tissue, such as easy accessibility of the highly organized retinal layers, the possibility to monitor treatment effects and relatively small size, have positioned

this organ at the forefront of gene therapeutic developments over the past 20 years. This is also the case for the current hot topic field of genome editing, which targets specific gene loci in order to repair

disease-causing mutations. Particularly in the retina, where mutations in more than 200 different genes can cause inherited retinal dystrophies [1], it seems that correcting a huge number of mutations is

highly promising. Most of the disorders originate in either the retinal pigment epithelial (RPE) cells or the photoreceptor cells, which have both previously been subject to the development of treatment approaches based on either viral vector-mediated gene therapy or cell-based therapy using re-differentiated stem cells or progenitor cells. Taken together, the premise is strong for the successful application of therapeutic genome editing for inherited retinal dystrophies; however, the question of whether the field has advanced far enough to become a clinical reality in the near future is intriguing. Important hurdles need to be overcome and advancing too rapidly may endanger the entire field.

Genome editing is based on the innate capacity of cells to repair DNA double-strand breaks (DSB), which are the most dangerous form of DNA damage that can occur to a cell [2]. By employing highly specific endonucleases, such as transcription activator-like nucleases (TALEN) or RNA-based nucleases (clustered regularly interspaced short palindromic repeats [CRISPR]-based systems) and in some cases a template DNA containing the sequence of choice, this technology can be efficiently used to modify the genome in any given cell [3]. Frequently occurring during mitosis in cases of a stalled replication fork or ionic radiation, DSBs are repaired either by: sticking the DNA ends together by a mechanism called non-homologous end-joining (NHEJ); use of the sister chromatid as template DNA via homology-directed repair (HDR) with long homologous regions; or via micro-homology mediated end joining (MMEJ) with

short homologous regions [4]. All DSB repair pathways depend on the cell cycle stage, with NHEJ being predominantly active in all stages and HDR and MMEJ being restricted to G2 or G1, respectively [5]. NHEJ, which generates insertions and deletions (indels) at the DSB site, has so far been used most often to knock out a given gene rather than to modify the genome in a defined manner in a therapeutic setting [6,7]. However, since NHEJ also seems to be the predominant DNA repair pathway in post-mitotic cells, researchers are actively looking for approaches in which NHEJ can be used without unwanted indel formation to repair a DSB in a defined manner [8]. In experimental approaches targeting HDR or MMEJ, the sequence to be repaired is presented as a template DNA with defined homologous regions in order to enable either HDR or MMEJ.

Overall, the development of therapeutic strategies currently leads in two directions: *ex vivo* genome editing and *in vivo* genome editing. *Ex vivo* approaches are based on the idea of taking a skin biopsy of the patient, de-differentiating the cells into induced pluripotent stem cells (iPSCs), correcting the mutation by gene transfer of endonuclease (and template DNA), re-differentiating selected cells into RPE or photoreceptor cells and re-implanting them into the retina [9]. The target cells re-enter cell division in the dedifferentiated state and can thus be easily treated, screened and selected for successful genome editing. However, the disadvantage of this approach is still the complex process of re-transplanting cells into the retina, which interact with the surrounding cells in a complex mechanism [10].

Alternatively, *in vivo* approaches aim to treat the mutations directly in retinal cells *in situ* [8,11–13]. There is no need for *ex vivo* de-differentiation, re-differentiation and re-implantation [14]. Vehicles for *in vivo* gene transfer exist in the form of virus-based vectors, such as adeno-associated viruses (AAVs), which are the current state-of-the-art vectors for efficient retinal gene transfer [15]. The major disadvantage here is, besides the technical issues related to the packaging size of viral vectors, the post-mitotic state of the cells, which very likely hinders efficient genome editing, and the absence of screening and selection possibilities, not to mention potential toxicity issues due to off-target activity associated with the CRISPR-Cas system. These points will be discussed in the following paragraphs.

The CRISPR-based endonuclease system comprises the Cas9 protein, which is led to the specific target site by a guide RNA [16,17]. The character of the guide RNA follows defined rules, such as the obligatory protospacer adjacent motif (PAM) sequence being adjacent to the 20 bp target sequence of the guide RNA [18]. The CRISPR-Cas9 protein often used in genome editing approaches originated from *Streptococcus pyogenes* (spCas9) and has a size of approximately 4 kB. Together with a promoter sequence and a polyadenylation signal, this expression cassette fills the entire loading capacity of AAV vectors, necessitating the presence of a second AAV comprising the expression cassette for the guide RNA and a potential template DNA. However, a two-vector system for the transfer of genes into the retina is very likely to be less efficient compared to an

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all-in-one vector. The introduction of the shorter *Staphylococcus aureus* Cas9 protein (saCas9) enabled the generation of single AAV vectors containing both the endonuclease and the guide RNA expression cassette [19,20]. Such a vector could be used for treatment approaches where NHEJ is envisaged and a template DNA is not necessary. The current treatment concept of Editas Medicine, targeting a splice site mutation in the *CEP290* gene, employs such a strategy in order to remove an additional splice site in one intron by indel formation. However, treatment approaches based on HDR or MMEJ, for which a template DNA is obligatory, single AAV systems are not possible if the endonuclease is based on the CRISPR system [21].

To overcome the size issue, several options are available, but none of them are as far advanced in the preclinical setting as the currently used AAV system [15,22]. Use of lentivirus- or adenovirus-based vectors have a larger packaging capacity, with 7 kBP or >20 kBP, respectively. However, the tropism of these vectors, together with the less optimal immunologic profile, renders them problematic as transfer vectors. Nanoparticles or the transfer of supercharged proteins also present an option for the transfer without a restriction of the size of the transferred material [23,24].

Short-term expression systems such as nanoparticles of supercharged proteins might be better suited than viral vectors to avoid unwanted off target toxicity.

However, efficacy of material transfer employing these systems is still not comparable with AAV vectors.

A few other issues need to be considered with regard to the transfer system. Application of AAV vectors to a cell seem to activate the DNA repair system on its own, potentially associated with the second strand synthesis following intranuclear transfer of the genetic information [15]. This feature might further increase repair efficacy in a therapeutic setting, again making this vector system more favorable compared to the other systems. However, another point might be even more important. In contrast to classic gene addition therapy, long-term expression of the transgene is not desirable in a genome editing setting. Rather, the endonuclease as well as the template DNA should only be present transiently in the target cell in order to avoid unwanted off target toxicity. Therefore, short-term expression systems such as nanoparticles of supercharged proteins might be better suited for such an approach than viral vectors. It remains to be seen which of the different vector systems will prevail and dominate the clinical application in the near future.

While experience from more than 20 years of experimental and clinical application of delivery systems will most likely help solve the transfer issues in the near future, almost nothing is known about the activity

of the DNA repair machinery in PR or RPE cells, be it human or murine retina. Most information about DNA repair proteins, the sensing of DSB and the subsequent cascades involved in DSB repair have been gathered from well-defined cell culture systems, in which all cells are in mitotic stages [5]. However, the post-mitotic state of neuronal retinal cells very likely hinders efficient genome editing in the retina. Together with the absence of screening and selection possibilities, this is the major drawback of *in vivo* genome editing.

A recent study nicely demonstrated that rod photoreceptors, which represent the majority of cells in the murine retina, behave differently to DSBs compared to any other cell type in the retina [25]. The group observed that adult rod photoreceptors repair only half of the induced DSBs within 1 day after damage induction by radiation, a defect that is not observed in any other cell type of the adult retina nor in rod photoreceptor precursor cells of postnatal day 4 mice, where almost all DSBs have been repaired within 24 hours. It is therefore absolutely mandatory to decipher the DNA repair mechanisms in mature photoreceptor cells in order to know exactly what is going to happen upon transfer of endonucleases for any genome editing application.

The observation that rod photoreceptors at day 4 have an efficient DNA repair system, as shown in the abovementioned study, indicates that there are differences in the behavior of developing photoreceptor cells compared to mature and differentiated cells. While at birth cells in the murine retina are still in a progenitor stage with a completely different gene expression profile,

this changes with eyelid opening around P13 as master regulators of neuronal gene expression change the global gene expression profile within the retina [26]. As a result, photoreceptors and other neurons differentiate into their final stage having a fundamentally different gene expression profile compared to cell populations at earlier time points around birth. Since targeted *in vivo* genome editing to repair disease-causing mutations is likely to take place in fully differentiated (i.e., mature) retinal neurons *in vivo*, research using embryonic or neonatal animal models are, therefore, not the optimal experimental setting in which to test highly specific endonucleases and the DNA repair capacity. The lack of useful systems besides the *in vivo* experimentation currently represents a substantial hurdle in the development of therapeutic applications.

Only a few *in vivo* applications have so far been published. In one study, inner retinal neurons, such as bipolar cells or ganglion cells have been shown to be able to repair artificially induced DSB following intravitreal application of an AAV vector in adult mice [11]. While this is the first *in vivo* application of the CRISPR-Cas9 system in the retina published so far, target cells were neither photoreceptor cells nor RPE cells, rendering the information of limited importance for inherited retinal dystrophies. A very recent study by Suzuki *et al.* demonstrated the application of NHEJ in a specific setting (i.e., homology-independent targeted integration [HITI]) to be useful in the targeted editing of the *MERTK* gene in a rat model of LCA [8]. In two other publications, the authors performed electroporation of plasmids containing the

genetic information of the endonuclease in neonatal mice and observed genome editing activity at 7 or 30 days post-treatment in mouse models of autosomal dominant RP associated with mutations in the rhodopsin gene [12,13]. However, as discussed previously, cells in the neonatal retina do possess an active DNA repair system that differs fundamentally from the situation in a mature retina, rendering the information from these papers only partly useful for later clinical applications in mature human retina.

In summary, while the expectations for genome editing to treat inherited retinal dystrophies are high, and a number of companies and research groups around the world are rapidly advancing towards clinical trials, fundamental questions have still only been partially solved and some remain completely unanswered, making this approach highly risky. Steps towards clinical application should be taken with great caution in order to prevent unwanted side effects that would hamper the development of the entire field, which would be a great disappointment for a large number of patients with a devastating, but not life-threatening disorder.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

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