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AAV-CRISPR Persistence in the Eye of the Beholder

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Despite advances in genome editing technologies based on the adeno-associated virus (AAV)-CRISPR system, there are still concerns about the long-term persistence of recombinant AAV vectors in several organs (liver, muscle, eye) possibly leading to cytotoxicity or genotoxicity related to off-target effects. Indeed, there are still unanswered questions about long-lasting *in vivo* AAV persistence as a linear or circular DNA that is not targeted by epigenetic silencing in many tissues. In 2017, Kim et al.[1] reported an editing approach based on AAV-CjCas9 to downregulate *Vegfa* or the hypoxia-inducible transcription factor *Hif1a* in mice displaying age-related macular degeneration (AMD)-related pathological choroidal neovascularization (CNV) induced by laser treatment. Although partial knockdown of either *Vegfa* or *Hif1a* provided benefits and reduced the area of CNV, local opsin dysfunction near the *Vegfa*-edited cells of murine retinal pigment epithelium (RPE) was observed. Conversely, no cone dysfunction was reported upon *Hif1a* partial knockdown. Lastly, no genome-wide off-target indels, evaluated 6 weeks after intravitreal injection of AAV-CjCas9 vector, were scored, indicating that prolonged expression of AAV-CjCas9 *in vivo* did not aggravate the genotoxic risk associated with the CjCas9 nuclease. In this issue of *Molecular Therapy*, the authors now report a long-term (14 months) safety study on C57BL/6J mice intravitreally injected with AAV-CjCas9 vectors targeting *Vegfa* or *Hif1a* genes.[2] The findings continue to show that the AAV-CRISPR system in the eyes is long lasting, effective, and safe.

CRISPR/Cas9 genome editing in the retina represents a potential treatment strategy for inherited retinal dystrophies (e.g., autosomal dominant retinitis pigmentosa [adRP] and Leber congenital amaurosis [LCA]) and retinal neovascular diseases (e.g., wet AMD and proliferative diabetic retinopathy). CRISPR components have been delivered to the retina by viral and non-viral methods. Although subretinal plasmid electroporation is not suitable for therapeutic interventions in patients, it has been employed to knock down a mutant Rhodopsin gene in mouse[3] and rat[4] models of adRP. Recently, preassembled *Vegfa*-specific Cas9 ribonucleoproteins (RNPs) have been subretinally injected into a mouse model of AMD, demonstrating a significant reduction of laser-induced CNV. However, the effects were localized only to the injected area of RPE, with no transduction of the neural retina.[5]

Nonvirally-mediated transient expression of CRISPR components in the retina may reduce safety concerns, although viral delivery systems based on AAV represent the most efficient and safe tools for gene delivery to the retina. Indeed genome editing using the AAV-CRISPR system has been widely reported as efficient, safe, and precise in more than 30 published studies in mouse models[6] of diseases associated with the eyes, muscle, liver, heart, and lung. Despite the great potential of AAV vectors, their relatively small packaging capacity represents a limitation for delivering the widely used *Streptococcus pyogenes* Cas9 (SpCas9) together with guide RNAs (gRNAs) and large transgenes. Dual-vector AAV systems, smaller Cas9 orthologs, or other nucleases belonging to the type-V

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CRISPR-Cas systems (AsCpf1 and LbCpf1) have circumvented the transgene packaging issue. Packaging of SpCas9 and gRNA into two separate AAV particles proved to be a successful delivery strategy.[7] Similarly, an AAV-split-Cas9 system has been developed. These strategies rely on the possibility to reassemble the Cas9 holoenzyme in transduced cells by fusing the N- and C-terminal domains of Cas9 to Rapamycin-inducible FRB-FKBP dimerization domains[8] or Intein, thus triggering *trans*-splicing of Intein-tagged Cas9 domains.[9] Smaller Cas9 orthologs from *Staphylococcus aureus* (SaCas9),[10] *Campylobacter jejuni* (CjCas9),[1] *Streptococcus thermophilus* (StCas9),[11] and *Neisseria meningitidis* (NmCas9)[12] have also been isolated. Owing to their smaller size (2.9–3.3 kb) compared to SpCas9, they potentially could be suitable for packaging into a single AAV vector also bearing the gRNA. However, StCas9 and NmCas9 have been neglected owing to their longer protospacer adjacent motif (PAM), which represents a limitation on sequences available for targeting. Conversely, SaCas9 and CjCas9 have been successfully vectorized together with gRNAs in a single AAV and show cutting ability comparable to SpCas9 in *in vitro* applications.[5] More recently, Cpf1 nuclease isolated from *Lachnospiraceae bacterium* (LbCpf1) has been vectorized together with the cognate CRISPR RNA (crRNA) into a single AAV vector,[13] demonstrating its great potential as an *in vivo* genome editing tool for the treatment of angiogenesis-related diseases.

AAV-CRISPR systems based on CjCas9 and LbCpcf1 nucleases have been developed to treat AMD, and AAV-CRISPR persistence and safety issues have been examined in mice.[14,15] Currently, patients with wet AMD benefit from intravitreal injection of anti-vascular endothelial growth factor (VEGF) agents (e.g., ranibizumab, bevacizumab, and aflibercept). However, there is a compelling need for a long-lasting therapy solution for patients with AMD because of the cost of the drugs, patient access to treatment, and the increasing number of patients as the population ages. In the new study, the authors showed no changes in histologic integrity and function of retinal tissues treated with AAV-CjCas9.[2] Then they reported an efficient editing in both target genes 6 weeks post-injection, which clearly increased 8 weeks later in both retina and RPE, although *Vegfa*-specific indels arose only in the retina. These data indicate the long-term persistence of constitutively expressed and active CjCas9 nuclease in the injected area. Lastly, the authors analyzed the potential off-target sites by targeted deep sequencing and performed a comprehensive genome-wide analysis of potential off-targets sites bearing up to 4 nucleotide mismatches in the mouse genome. Any detectable indels were scored in 21 homologous sites. Altogether, this study demonstrated that intravitreal injection of AAV-CjCas9 vector targeted to *Hif1a* effectively induced and maintained mutations in murine retinal cells for 14 months without giving rise to off-target indels due to the constitutively active CjCas9 nuclease and without affecting retinal histologic integrity or function. Unfortunately, no data on CNV amelioration 14 months post-injection are reported. It would have been extremely useful to see whether higher indel frequency at target sites resulted in more effective treatment of wet AMD.

Other AAV-CRISPR systems developed for treatment of adRP or LCA 10[7,16–18] showed prolonged and safe expression (up to 9.5 months) of subretinal-injected AAV-SpCas9 targeting specific mutations in *RHO* or *CEP290* genes or targeting neural retina leucine zipper transcription factor (Nrl) in mouse models for adRP. Although, in these studies, the authors employed different AAV serotypes and different vector doses and targeted different genes, the results showed effective rescue of RP or LCA 10 phenotype without toxicity or off-target effects. We now have a potent molecular tool to employ in several eye disorders because Kim's group demonstrated a robust *in vivo* knock down of a murine transcription factor, perfectly conserved in the human genome and involved in tumor angiogenesis and pathophysiology of ischemic disease, and maybe this strategy could be translated to other vascularization-associated human diseases.

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The Bumpy Road to CAR Activation

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A rapidly emerging immunotherapy approach is the use of chimeric antigen receptor (CAR) T cells. While adoptive transfer of CAR T cells has seen striking results in some malignancies, its efficiency has been tempered in others due to limited expansion, persistence, and tumor homing of the transferred cells.[1] Another clinical limitation is the serious toxicities that may arise following CAR T cell tumor recognition (reviewed in Sadelain et al.[2]). This raises a very important issue that must be resolved for proper cell engineering: are we fully harnessing the biology of artificial T cell signaling for effective immunotherapy? A recent paper in *Science Signaling* by Salter et al.[3] demonstrates that the signaling cascades initiated by synthetic CARs cannot be predicted entirely by their design and sheds light on the differences between the phosphorylation of the proteomes of CD28/CD3ζ compared to 4-1BB/CD3ζ CAR T cells.

The last decade has seen the emergence of antitumor therapeutic development. The Nobel Prize in Physiology or Medicine 2018, awarded to James P. Allison and Tasuku Honjo, has added to the enthusiasm toward immunotherapy. Their work has created a major shift in our understanding of the immune system recognition of malignancies and how we can manipulate this new weapon to our advantage. We are indeed entering a new era where immunotherapy will soon complement standard radiotherapy or chemotherapy regiments for cancer treatment.

CAR T cells are engineered T cells expressing fusion proteins, mostly combining an antigen-specific single-chain fragment (scFv) coming from a monoclonal antibody with T cell receptor (TCR) intracellular signaling domains. *In vitro* studies have demonstrated that first-generation CAR T cells, containing only a CD3ζ moiety, support T cell activation and target cytotoxicity, but with very limited persistence and antitumor efficacy following adoptive transfer.[4] Second-generation CARs, therefore, incorporated a two-signal model of T cell activation by modifying the CARs to include a CD28 or 4-1BB (CD137) costimulatory domain that provides signals for T cell effector function, proliferation, and, more importantly, persistence.[5,6] Nevertheless, in recent years, these CAR constructs have shown variable effects *in vivo*. Therefore, the authors of the new study aimed to assess whether differences between CD28/CD3ζ and 4-1BB/CD3ζ CAR T cells were attributable to divergent T cell activation pathways. Human primary T cells were transduced with modified lentiviral vectors encoding CD19- or ROR1-specific CD28/CD3ζ CARs and identical CD19- or ROR1-specific 4-1BB/CD3ζ CARs fused to a nine-amino acid Strep-tag II (STII) sequence in the extracellular CAR hinge. Canonical T cell signaling events were then evaluated following STII microbead stimulation. Using an elegant phosphoproteomic approach with liquid chromatography-tandem mass spectrometry (LC-MS/MS), they identifed many novel phosphoprotein signaling events in stimulated CARs that were not identified using previous methods. Of note, both CD28/CD3ζ and 4-1BB/CD3ζ CARs could promote phosphorylation of endogenous CD28 following stimulation. Surprisingly, they concluded that patterns of protein phosphorylation were very similar in cells expressing either of these CAR

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