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Commentary

From Puppies to adults: *In vivo* editing of hepatocytes in a canine model of glycogen storage disease type Ia

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Transgene expression after adeno-associated virus (AAV)-mediated gene therapy would need to last for decades to treat most genetic disorders, especially those that need hepatic correction. However, conventional AAV gene edition approaches rely upon on episomal transgenes for expression, which are known to decrease in the liver over time.[1] To address the problem of therapeutic durability, *in vivo* genome editing of hepatocytes has been pursued as a strategy to enable permanent transgene expression.[2] Recently, Arnson et al. demonstrated genome editing and long-term hepatocyte correction in a canine model of glycogen storage disease type Ia (GSD Ia) following AAV treatment.[3] While gene and base editing has shown promise in murine models of GSD Ia, the successful application of this approach in a large animal model is an important next step for potential translation to GSD Ia human patients and an extension of this approach to other metabolic disorders.

GSD Ia is a severe metabolic disorder caused by the deficiency of glucose-6-phosphatase (G6PC). The clinical symptoms are dominated by recurrent, and potentially fatal, episodes of hypoglycemia caused by impaired glycogenolysis. The current treatment for GSD Ia requires dietary supplementation with cornstarch to maintain blood glucose homeostasis, medical management of disease-related systems, and liver transplantation in patients who do not respond to dietary management.[4] Patients with GSD Ia also develop hepatic adenomas and require lifelong monitoring for hepatocellular carcinoma, a recognized long-term complication of the disorder. The difficult clinical course, challenging dietary management strategy, and guarded long-term outcomes for patients has led to the development of alternative treatments, including AAV gene therapy, which is the subject of a recent phase 3 clinical trial (https://clinicaltrials.gov/ct2/show/NCT05139316 Identifier: NCT05139316).

Early studies in GSD Ia mice and canines using canonical AAVs designed to express *G6PC* in the liver resulted in a short-term improvement in the disease phenotypes followed by a relapse in disease due to loss of episomal transgenes.[5] This led to studies in murine models where phenotypic correction could be achieved by genome editing strategies with zinc-finger and CRISPR-Cas9 nucleases to incorporate the transgene into the mouse genome, resulting in stable long-term transgene expression.[6,7] In addition, it was noted that bezafibrate could increase gene editing and transgene expression by stimulating autophagy.[8] Whether gene editing for GSD Ia would provide therapeutic benefit in a large animal model, as assessed by fasting tolerance, was the main question that the team from Duke University sought to address.

The GSD Ia editing approach in canines used two AAV vectors: a therapeutic AAV donor vector with a promoter and a *G6PC* transgene to target the *G6PC* locus by homologous recombination (HR). The promoter was included in the donor vector to allow for transgene expression of both integrated and non-integrated therapeutic vectors (Figure 1). A second AAV vector encoded an SaCas9 nuclease and a guide RNA (CRISPR-SaCas9) designed to cut the *G6PC* locus at the location of HR to increase the frequency of this event. Both the CRISPR-SaCas9 and the donor vector were packaged in an AAV7 capsid. Prior to *in vivo* testing, these AAV vectors were tested in canine fibroblasts and demonstrated the ability to generate the desired edited allele. Two GSD

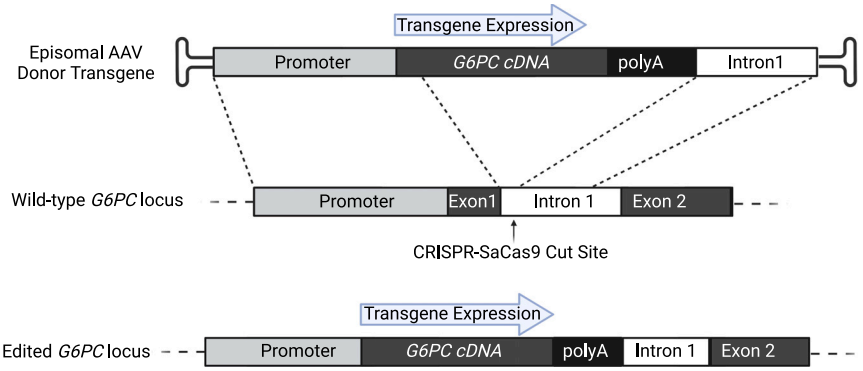

Figure 1. Schematic of donor AAV vector genome and edited canine *G6PC* locus

The 5′ prime homology arm of the donor vector contains the endogenous *G6PC* promoter enabling expression of the *G6PC* cDNA from episomal (non-integrated) vector genomes, and the 3′ homology arm targets intron 1 and contains a mutated PAM site (not depicted). The guide RNA targets the intron 1 for CRISPR-SaCas9 cleavage. Created with BioRender.com.

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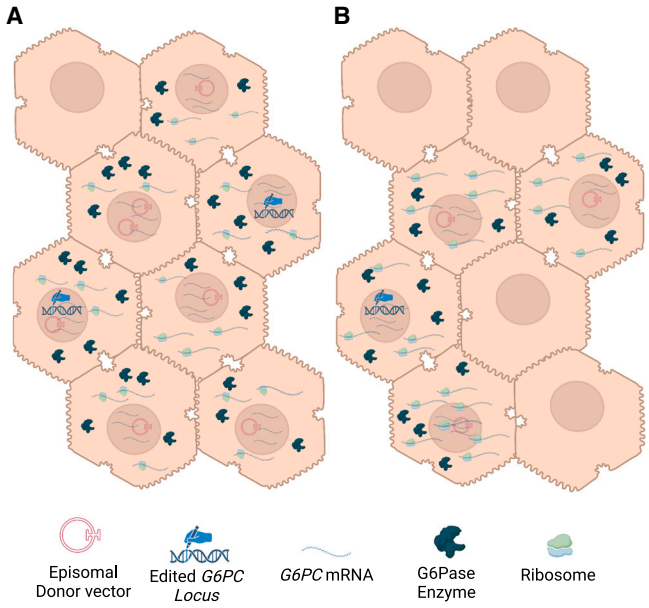

Figure 2. Transgene expression from donor episomes and edited *G6PC* loci over time with GSD Ia genome editing strategy

(A) Shortly after transduction, the donor vector exists predominately as episomes, and only a fraction of the therapeutic donor vector is integrated in the *G6PC* locus. During this time, a majority of the *G6PC* expression is coming from episomes with only small amounts of *G6PC* expression coming from hepatocytes with edited *G6PC loci*. Note, the CRISPR-SaCas9 AAV vector (not depicted) exists as an episome and was not designed to integrate into the genome. (B) As hepatocytes divide and die, the number of hepatocytes that contain episomes is substantial reduced, while the edited hepatocytes persist, albeit in small numbers, and continue to express the therapeutic transgene. Created with BioRender.com.

Ia pups (2 days old) and three GSD Ia adult canines (34 months old) were treated with the AAV7 donor and CRISPR-SaCas9 vectors and evaluated for 16 months post-injection. In addition to the gene editing AAV vectors, canines received bezafibrate to increase autophagy, which was shown to increase correction in a murine study and conventional AAVs to prevent a potentially lethal crisis, which allowed for long-term follow-up after gene editing. After treatment with the AAV7 CRISPR-SaCas9 and donor vectors, there was evidence of gene editing in the form of HR from transgene integration (<1%) and indel formation from cutting and non-homologous end-joining at the *G6PC* locus in both neonatal and adult treated GSD Ia canines. Canines treated as pups and adults displayed increased transgene expression and G6PC enzymatic activity, reduced hepatic glycogen storage, and improved glucose regulation. However, since the episomal donor AAV expressed the transgene and all of the canines were treated with conventional AAVs, it is difficult to deduce to what extent the transgene expression from edited hepatocytes contributed to improvement observed, but genome editing alone was not sufficient to prevent lethality. Additionally, the inclusion of the endogenous promoter in the donor vector allowed the treated GSD Ia pups to survive longer than untreated pups, which likely was due to transgene expression from episomal donor vectors near treatment. In addition, the treated GSD Ia pups required additional treatments with conventional AAV vectors for survival to the end of the study due to the reduction in transgene expression over time (Figure 2).

Notably, while toxicity was not a focus of this study, none of the treated canines had any findings that were not related to GSD Ia. However, SaCas9 protein was detectable in canines at 4 months post-treatment, and the persistent expression of a nuclease does raise some potential safety concerns, such as the increased potential for off-target cutting and an immune response to this exogenous protein. The use of mRNA lipid nanoparticles has been suggested to alleviate safety concerns that might arise from long-term expression of nucleases. Since this approach utilizes the endogenous *G6PC* promoter, the risk of toxicity from transgene overexpression and genotoxicity from the use of strong exogenous enhancer and promoter are reduced.[9]

An ongoing GSD Ia AAV8 gene delivery study that uses a conventional episomal transgene approach for transgene expression has reported patient improvement, but it is still unclear how long after gene delivery a therapeutic benefit might last.[10] Treatment of GSD Ia in earlier childhood would yield the greatest benefit for patients, but this is also the time period where the loss of episomal transgenes in the liver is most problematic, and stable transgene expression from edited hepatocytes might be necessary. But further refinements in genome editing for GSD Ia to achieve higher levels of editing likely will be needed to make gene editing for GSD Ia a standalone therapy and might include the use of AAV capsid with greater liver tropism, higher doses of the donor vector, the delivery of the nuclease as mRNA lipid nanoparticle, and the creation of guide RNAs with better on-target cutting efficiency. However, the results from the ongoing clinical trial are encouraging for the genomic editing approach used in the GSD Ia canines, because it also allows for episomal expression and has the added benefit of long-term transgene expression from edited hepatocytes.

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DECLARATION OF INTERESTS

The author declares no competing interests.

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