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Insertion of Short Double-Stranded Oligonucleotides Using CRISPR/Cas9 as a Therapeutic Approach for Glycogen Storage Disease Type 1a

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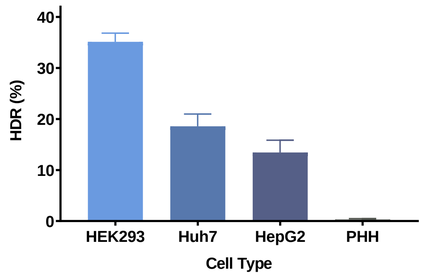
Abstract

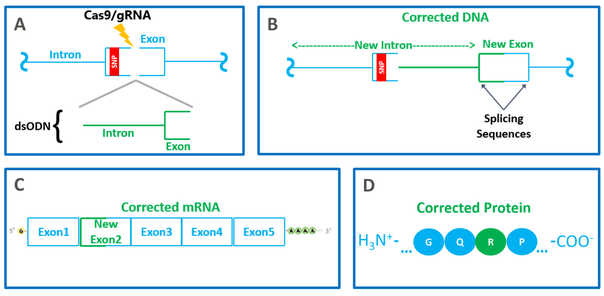
Glycogen storage disease type 1a (GSD1a) is a rare autosomal recessive disease that can lead to life threatening hypoglycemia and progressive liver and kidney damage. GSD1a is caused by loss of function mutations in the glucose-6-phosphatase-α (G6PC) gene. G6PC, an integral membrane protein primarily expressed in the liver, kidneys, and intestines, enzymatically cleaves phosphate from glucose-6-phosphate to liberate glucose to stabilize blood sugar levels. Here, we explore CRISPR/Cas9-based strategies to correct the prevalent R83C mutation in G6PC by utilizing non-homologous end joining (NHEJ) repair mechanisms and short double-stranded DNA oligonucleotides (dsODNs). We identified guide RNAs (gRNAs) that cleave G6PC exon 2 genomic DNA near the site of the mutation and for each gRNA, designed dsODNs to introduce the R83C mutation as a proxy for correction. Each dsODN, when transcribed to RNA, consists of a 3′ splice site (a branch point sequence, a polypyrimidine tract, and a terminal YAG) and an exonic region containing protein coding sequences. Insertion of the dsODN creates a new version of G6PC exon 2 that has tandem 3′ splice sites and a single 5′ splice site. As predicted by the exon definition model of splice site pairing, the newly introduced 3′ splice site is proximal to the 5′ splice site and spliced to G6PC exon 1, resulting in the protein coding sequences from the dsODN being included, in frame, in the final mRNA. The introduction of Cas9 mRNA, a single-stranded gRNA, and the dsODN into primary human hepatocytes (PHHs) *in vitro* via transient transfection and into mice *in vivo* via lipid nanoparticles routinely led to over 10% of mRNA isolated from both mice and PPHs containing the R83C mutation as measured by Next-Generation Sequencing. Of note, studies in mice have suggested that restoring approximately 4% of G6PC activity could prevent the hypoglycemic seizures that occur in GSD1a patients. The dsODN insertional technique described here bypasses the need for the homology-directed recombination machinery and functions in both dividing and non-dividing cells, making this technique an intriguing potential means to correct a number of genetic diseases in cells and tissues that have low rates of homologous recombination.

Figure 1: Non-Dividing Cells Lack Homology Driven Repair



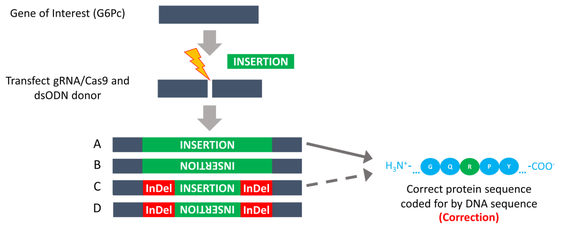
The rates of homology dependent repair (HDR) in dividing and non-dividing cells. Dividing (HEK293, Huh7, and HepG2) and non-dividing cells (primary human hepatocytes (PHHs)) were transiently transfected with *S. pyogenes* Cas9 mRNA, a single-strand guide RNA (sgRNA) specific to G6Pc exon 2, and a single-stranded oligonucleotide HDR template. 48 hours post-transfection, genomic DNA (gDNA) was isolated and Next Generation Sequencing (NGS) was performed and analyzed to determine the percentage of alleles that had undergone full or partial HDR.

Figure 2: Gene Correction Using NHEJ to Insert a Short Double-Stranded DNA Oligonucleotide



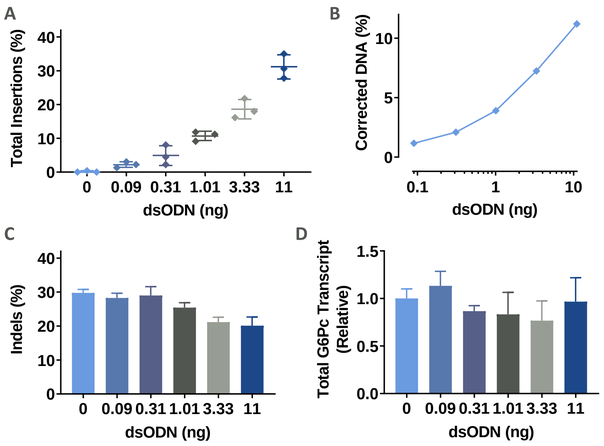
Schematic representation of correction strategy. (A) A double strand DNA break is introduced at a specific location in the G6PC exon 2 by Cas9 bound to a gRNA. A short dsODN that incorporates both a 3′ splice site and exonic sequences that are corrective for the R83C mutation associated with GSD1a and additional silent mutations is inserted into the break by the NHEJ machinery resulting in the new genomic architecture depicted in **(B)**. The model of exon definition predicts that the newly inserted 3′ splice site will be preferentially used over the original 3′ due to the proximity to the 5′ splice site. Utilization of introduced 3′ splice site will lead to mRNA lacking the R83C mutation due to RNA splicing **(C)** and the correct protein sequence **(D)**.

Figure 3: Expected DNA Structures from Insertion of the dsODN



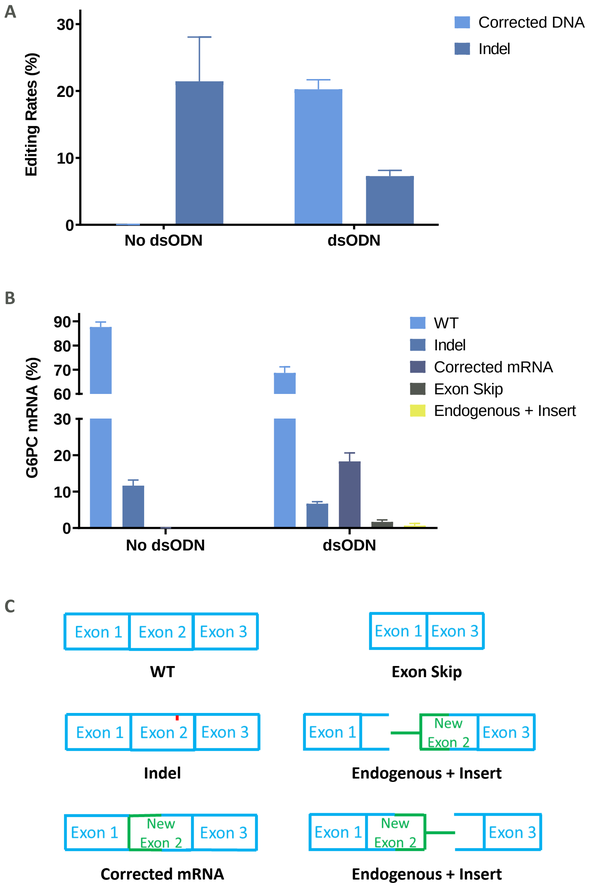
Genomic implications from insertion of dsODN in exon 2 of G6PC. The insertion of the short dsODN into a Cas9-mediated dsDNA break can lead to several outcomes. **(A)** Insertion of the dsODN in the desired orientation without the presence of insertions or deletions (indels). **(B)** Insertion of the dsODN in the reverse orientation without the presence of indels. **(C)** Insertion of the dsODN in the desired orientation with the presence of indels. **(D)** Insertion of the dsODN in the reverse orientation with the presence of indels. Of note, the outcomes described in both **(A)** and **(C)** can lead to the formation of functional mRNA and protein (correction). Addition of bases on the intronic side of the dsODN will not affect RNA splicing.

Figure 4: NHEJ Driven Insertion of dsODN in Non-Dividing Primary Human Hepatocytes



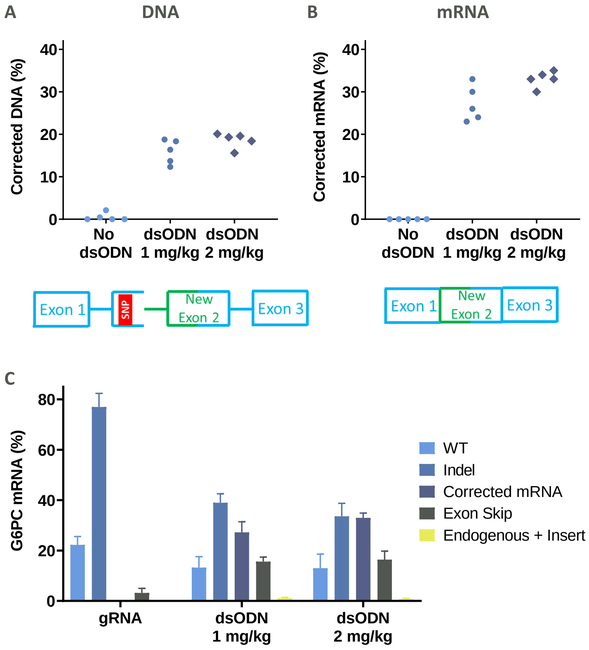
"Correction" in PHH. Cas9 mRNA, a G6PC-specific gRNA, and increasing amounts of a dsODN containing a 3′ splice site and G6PC coding sequence up to the site of Cas9 cleavage harboring silent mutations were transiently transfected into PHHs. 48 hours after transfection gDNA was isolated and subjected to NGS and analysis. **(A)** Total insertions were calculated as a percentage of all sequences that contained the dsODN regardless of orientation or indels. **(B)** "Corrections" were calculated as the percentage of total reads that had the dsODN inserted in the correct orientation without indels as well as the percentage that inserts in the correct orientation and had indels on the intron half of the dsODN. **(C)** "Indels" are shown as the percentage of NGS reads that displayed indels but lacked inserts. **(D)** qRT-PCR was performed on total RNA to determine the amount of the G6PC transcript present 48 hours after transfection.

Figure 5: Splicing Phenotype in Edited Primary Human Hepatocytes



The inserted 3′ splice site is preferentially used in PHHs. Cas9 mRNA and a G6PC-specific gRNA were transfected with and without the dsODN. 48 hours after transfection total mRNA and gDNA was isolated and subjected to NGS and analysis. **(A)** Indels and "corrective" insertions were calculated as in **Figure 4** from gDNA. The splicing phenotype was determined by examining mRNA species **(B and C)**. The species of mRNA include: WT—wildtype mRNA from the G6PC locus, Indel—wildtype mRNA with the addition of an indel at the Cas9 cut site, "Correction"—mRNA harboring the silent mutations present in the dsODN, Exon Skip—transcripts missing exon 2 of G6PC, and Endogenous + Insert—transcripts using the naturally occurring 3′ splice site and having the dsODN represented in the mRNA.

Figure 6: Lipid Nanoparticle Delivery of Cas9, gRNA, and dsODNs for *In Vivo* Insertion into Mouse Liver



Silent mutation in G6PC mRNA were used to follow dsODN insertion in mouse hepatocytes. Cas9 mRNA, a G6PC-specific gRNA, and a dsODN were formulated into lipid nanoparticles (LNPs). 8-week old mice were intravenously injected with LNPs containing mRNA and gRNA. Two groups received either 1 mg/kg or 2 mg/kg of dsODN. 7 days after injection the animals were sacrificed, and the livers harvested for RNA and DNA analysis. **(A)** "Corrective" insertions were calculated as in **Figure 3** from gDNA. The splicing phenotype was determined by examining mRNA species **(B and C)** as in **Figure 5**.

Conclusions from Preclinical Studies

- HDR is not a viable path for editing non- or slowly dividing cells
- NHEJ insertion of a short dsODN occurs at therapeutically relevant levels at Cas9-mediated dsDNA breaks in non-dividing cells *in vitro*
- The inserted sequences can drive splicing to introduce desired sequences into functional mRNA
- Therapeutically relevant levels of editing have been demonstrated using LNPs to deliver Cas9, gRNA, and dsODN *in vivo* in mice
- dsODN insertional correction has the potential to be a therapeutic for Glycogen Storage Disease Type 1a