

Gaucher's disease treated via rAAV gene therapy

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Approximately one in every 40,000 newborns each year suffer from Gaucher's disease worldwide, which equates to roughly 3,500 children annually. Gaucher's disease is a part of the lysosomal storage disease family and is a genetic defect that results in the buildup of glucocerebroside, a glycolipid, in the liver, spleen, and bone. This accumulation is due to a lack of the enzyme glucocerebrosidase which breaks down and recycles the glycolipid. As standard care for Gaucher's disease, enzyme replacement therapy has been used to balance the high levels of glucocerebroside with a modified version of the typical enzyme glucocerebrosidase. Although this is an effective and reliable treatment, patients must receive the enzyme via intravenous infusion (IV) every two weeks, which may become extremely inconvenient at times due to its high maintenance. To combat this need and establish a definitive treatment of Gaucher's, we plan to engineer a recombinant adeno-associated viral (rAAV) vector to stimulate the production of glucocerebrosidase within the body.

Keywords: Gaucher's disease, gene therapy, glucocerebroside, glucocerebrosidase, AAV

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Background

As one of the most common lysosomal storage disorders, patients with Gaucher's disease suffer from the inability to produce glucocerebrosidase, resulting in a buildup of glucocerebroside, a glycolipid, in the spleen, liver, and bone marrow. There are three types of Gaucher's disease, with type 1 being most prevalent (around 95 percent of cases) compared to types 2 and 3. Symptoms range from the enlargement of the liver and spleen, blood cell abnormalities, and in some cases, severe neurological problems that lead to death. Currently, there is no definite cure for Gaucher's disease, but there are temporary solutions. Treatment options for types 1 and 3 include enzyme replacement therapy via

prescription or intravenous infusion every two weeks, and treatment for type 2 has yet to be developed due to the shortened lifespan of those affected by it. Unfortunately, the greatest drawback to the use of enzyme replacement therapy is its high cost. For prescription medications and intravenous infusion, the average cost per year equates to approximately \$275,000 to \$550,000 (Beutler 1991).

Many diseases, including Gaucher's disease, are caused by the defective expression of proteins due to mutated, modified, and missing genes. In this case, more than 380 mutations in the glucocerebrosidase (GBA) gene cause Gaucher's disease (U.S. National Library of Medicine 2018), with most of them being point mutations

in beta-glucocerebrosidase, altering the composition of the enzyme. The gene encoding for glucocerebrosidase is located on chromosome 1 in the region of q21. To address the issue at hand, our gene therapy approach utilizes modified adeno-associated virus (AAV) vectors to deliver restorative genes to cells at the mutated source. Due to AAV's single stranded DNA capacity, we can easily replace the viral DNA with new DNA that will promote the production of glucocerebrosidase in our patients. By supplementing the vector with the correct version of the mutated gene, we can utilize the precisely coded vector to deliver necessary copies of the desired gene to specific organs or tissues.

In fact, AAV vectors have become the dominant method of gene therapy for genetic diseases and have seen success in the treatment of spinal muscular atrophy for infants (Onasemnogene abeparvovec) and inherited retinal diseases caused by mutations in both copies of the RPE65 gene (Voretigene neparvovec). Unlike regular viruses, AAV vectors are unable to reproduce without an outside source, which allows scientists to control the amount administered to the patient. To date, AAV has not been associated nor known to result in human disease. In terms of efficiency, AAV has demonstrated its practicality through a range of favorable attributes as well. For one, it has the ability to transduce dividing and nondividing cells and confer long lasting transgene expression after a single injection.

Systems level

As of now, there have been eleven serotypes of AAV identified. Each of these serotypes vary in their tropism (natural inclination to specific organs) and the types of cells they infect. The plan we designed involves the use of AAV8 as the optimal serotype due to its tropism for the liver. Our idea is to engineer a specific recombinant AAV8 vector directed to the liver to trigger production of glucocerebrosidase in the body.

The AAV genome is flanked by 145-nucleotide inverted terminal repeats (ITRs), which are necessary for packaging and site-specific integration (Manfredsson 2016). The DNA elements required for replication and packaging of rAAV are the ITRs which flank the transgenic DNA in the cis gene, the rep/cap genes, and the adenovirus helper factors. To produce our construct, we utilize helper plasmids and a specific packaging cell line, HEK293. This permanent cell line, established from the primary embryonic human kidney, expresses a number of adenoviral genes which can be used to propagate adenoviral vectors.

Our treatment relies on the functionality and efficiency of an engineered rAAV vector treatment to combat

Gaucher's disease. The treatment will be introduced into the body through a peripheral vein near the liver. The rAAV8 construct will then bind to the 37/67-kilo Dalton laminin host-cell receptor (Nam, Lane, Padron, et al. 2007) and introduce our gene of interest, GBA, into the genome. Once the infected cell begins expressing the gene, we should see an increase in production of glucocerebrosidase and thus a decrease in accumulation of glucocerebrosidase in the body.

Device level

Due to the size of the gene encoding for glucocerebrosidase and the limited packaging capacity of rAAV vectors, we decided to utilize an overlapping dual rAAV vector approach to merge our large transgene into a single vector (Figure 1). The approach works like this: the cDNA of the GBA gene is split such that one vector contains a GBA-specific promoter and a part of the gene, and another vector contains the second part of the gene and a poly-A tail. Each vector will also contain a homologous sequence overlap of the GBA gene to ensure homologous recombination. The result should be a large reconstructed transgenic cassette containing a GBA gene specific promoter, GBA cDNA, and a poly-A tail flanked by the inverted terminal repeats.

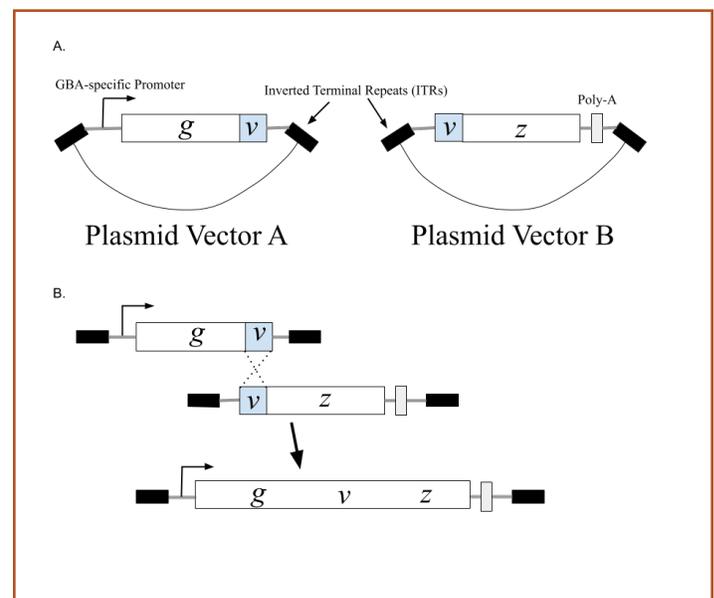


Figure 1. (A) The transgenic cassette is split into two plasmid vectors such that there is a homologous sequence overlap. Vector A contains our GBA-specific promoter, split cDNA sequence, and homologous sequence overlap. Vector B contains the homologous sequence overlap, remainder of split cDNA transgene, and a poly-A tail, which promotes transcriptional longevity in eukaryotic cells. (B) Following the construction of vector genomes A and B, homologous recombination at the overlapping sequence occurs, producing the large transgenic cassette.

After successful co-transduction of the vector genomes, our next step is to propagate the rAAV vector via triple-plasmid transfection into HEK293 cells (Figure 2), in which each plasmid contains either the rAAV genome with the cDNA for the GBA gene, the rep/cap genes, or the adenovirus helper factors. For our helper plasmids in the triple-plasmid transfection, we will be using pAAV2/8 and pAdDeltaF6. The pAAV2/8 plasmid was chosen for its specificity towards the AAV8 vector and its expression of the necessary rep/cap genes which produce proteins required for the AAV8 life cycle. The pAdDeltaF6 plasmid was chosen for its efficiency in AAV packaging, as well as for expressing the adenovirus E4, E2A, and VA factors, which function by promoting AAV protein production and altering the cellular environment to promote replication. Upon transfection, the HEK293 cell line will begin producing mature AAV8

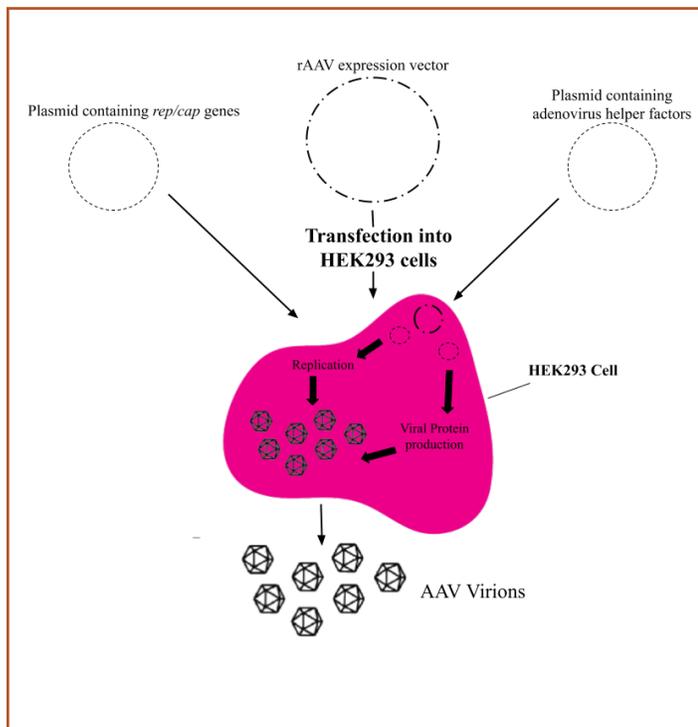


Figure 2. In the triple transfection approach, three plasmids, each containing either the rAAV expression vector with our transgene, the adenovirus helper factors, or the rep/cap genes are transfected into our production cell line, HEK293. The cell line then produces mature rAAV8 capsids containing our gene of interest, GBA.

capsids with our gene of interest (GBA) which will then be purified on a Cesium Chloride (CsCl) density gradient that separates the engineered AAV8 vector from contaminants such as cell debris and empty rAAV8 vectors based on their buoyant densities to a vector purity of >99% (Strobel, Miller, Rist, et al. 2015).

Parts level

We have created a theoretical design and protocol to express our desired gene, GBA, in humans suffering from Gaucher's disease. As aforementioned, we will be utilizing an overlapping dual rAAV vector and triple plasmid transfection approach to produce our rAAV8 construct. The parts used to do so include the pAdDeltaF6 plasmid, which was chosen for its capability in AAV packaging through its expression of the E4, E2A, and VA adenovirus helper factors, the pAAV2/8 plasmid, which was chosen for its expression of the rep/cap genes necessary for the packaging of the AAV8 capsid, and lastly, the AAV8 serotype due to its strong affinity towards the liver. Additionally, we chose to transfect the plasmids into the HEK293 cell line because it already contained two adenovirus helper factors, E1A and E1B, that pAdDeltaF6 did not provide.

Safety

Although the biosafety level (BSL), or pathogen protection level, of AAV vectors is level 1 (UNC School of Medicine 2020), handling human cell lines (HEK293) calls for biosafety level 2 practices and containment. This applies to laboratory settings typically taking place on benches without the use of special containment equipment. When working with agents associated with human diseases, enhanced measures must be taken due to the potential risk of the infectious microbes. Those working in BSL-2 labs are expected to take greater care to prevent injuries of the skin, mucous membrane exposures, and ingestions. Of course standard microbial practices are taken into account as well: mechanical pipetting only, daily decontamination, and proper personal protective equipment. Procedures that may cause droplets involving the HEK-293 cell line should be handled in a Class II biosafety cabinet (BSC). When working outside the BSC, wearing a full face shield is recommended.

Whenever a spill accident occurs, BSL-2 labs require immediate decontamination (Trapotsis 2020). In terms of handling, AAV cultures will be purified in phosphate buffered saline at a concentration of 10¹² particles/ml. The purified viral vectors will be stored for short periods of time at -20 or 4°C, in a freeze/thaw cycle. Low protein binding tubes will be utilized to avoid loss of the virus.

Since our design is proposed as a treatment for humans, there are several precautions we will take to ensure proper production. We will focus on testing our construct both in vivo and in vitro to optimize the treatment. While studies show that usage of mice, both immunized against the AAV capsid and not, for AAV testing failed to predict

the issue related to the T-cell reactivity to the capsid in humans (Colella, Ronzitti and Mingozzi 2017), they provided to be an extremely valuable assessment to AAV safety and efficacy.

Discussions

We recognize that performing an overlapping dual vector approach to produce our large transgene may have implications regarding the effectiveness of our AAV8 construct. However, a study published in Nature Biotechnology suggested otherwise, and demonstrated that when testing in vivo, the best performing overlapping dual vector performed similarly to a single gene comparison (Halbert, Allen and Miller 2002).

Currently, gene transfer utilizing AAV vectors on the liver has been tested clinically for a low number of diseases. In fact, a study in 2017 intravenously administered an scAAV8 (self-complementary) vector targeting the liver of hemophilia B subjects (Colella, Ronzitti and Mingozzi 2017). According to this study, immunosuppression was used to block potentially harmful immune responses stimulated by the viral vector. The study supports the idea that targeting the liver via injection through the peripheral vein with the AAV8 serotype is plausible.

Despite this, questions remain unanswered for the future of therapeutic AAV gene transfer in humans. One example involves immunogenicity, the tendency to trigger an unwanted immune response due to a foreign substance. Composed of both a protein capsid and a DNA genome, AAV vectors' viral capsid is nearly identical to the capsid of the "wild-type" virus which humans are exposed to in nature. A recent study conducted in 2019 screened 49 patients for pre-existing neutralizing antibodies and found that 15 of them (30.6%) had AAV8 specific antibodies (Aronson, Veron, Collaud, et al. 2019). The presence of neutralizing antibodies in the body will definitely impact the design of our construct and future clinical trials.

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