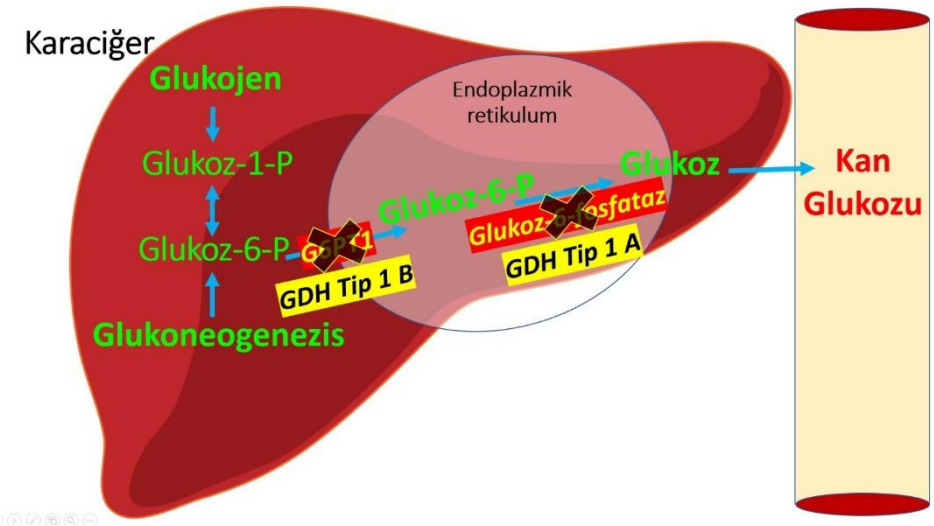
INTRODUCTION

Description of Disease

Glycogen storage disease is an autosomal recessive disease characterized by dysfunctional carbohydrate metabolism due to the absence or insufficiency of enzymes responsible in the synthesis or release of glycogen. GSD subtypes have been described according to the localisation of the disrupted enzymes in the metabolic pathway.

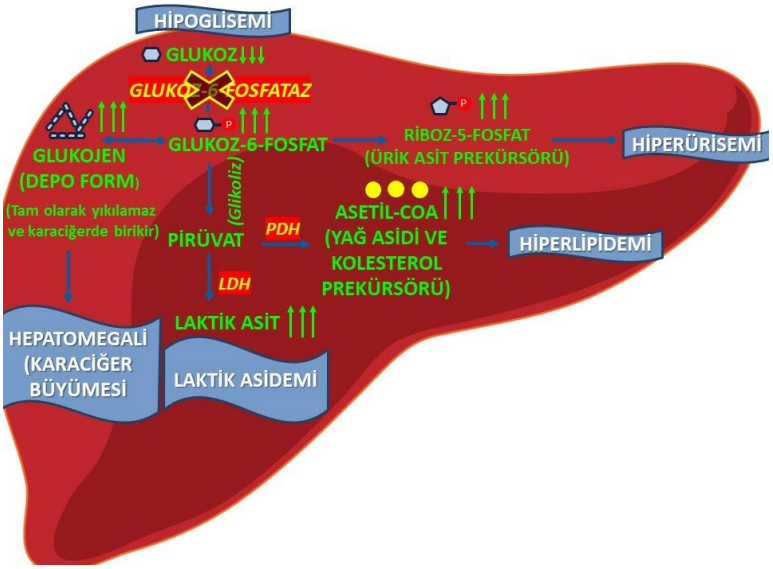
The most common of all GSD types is GSD type 1, with an incidence of 1/100,000 per live birth [1]

GSD Type 1A is caused by the absence of the glucose-6-phosphatase (G6PC) enzyme, which is required for the conversion of glucose-6-phosphate to glucose; GSD Type 1B is caused the absence of the G6P transport protein (G6PT1). Of all GSD Type 1 patients, 80% belong to the Type 1A subtype.[2]



These proteins are essentially active in the liver and kidneys. [3] Due to the storage of the excess glucose as glycogen and the inability to convert the glycogen to glucose during hunger, a drop in blood glucose levels (hypoglycemia) is seen, which may lead to developmental deficiencies. As a result of the excess storage of glycogen in the liver, the liver may be enlarged, causing abdominal swelling. Aside from these effects, the glucose-6-phosphate, which could not be converted to glucose, will take part in other pathways to cause an increase in uric acid, lactic acid and triglyceride levels. Patients usually apply to the hospital with these symptoms in the first 3-6 months postpartum.[4]

The definitive diagnosis is made with genetic tests.



The disease does not have a cure in the current day. Patients are followed up with diets to preserve physiological blood glucose levels. Continuous tracking of blood glucose levels are crucial for the prevention of hypoglycemia, during which fatal conditions such as seizures may occur aside from relatively mild symptoms such as fatigue, irritability and discomfort. [5] Furthermore, GSD patients have the risk of developing liver adenomas in 20-30 years of age, which may bleed and also carry the potential for malignancy. [6]

For this reason, it is very important for a definitive cure to be developed for GSD.

The project was designed to help these people required to fight this disease right after the first few months of their lives. “Primum non nocere” or “Do not harm” as was thought in medical school to us has been the primary principle in the design of this project. The treatment model in all stages were designated according to said principle.

Integrase Defective Lentivirus (IDLV)

The defect in the glucose 6-phosphotase gene (G6PC) is responsible for the occurrence of GSD Type 1A disease. Over 100 mutations have been discovered on this gene to this date and the prevalence of these genes in different races shows variance. [2] [7]

As it is not effective to treat every single gene mutation separately, it was aimed to carry out the treatment by transferring the functional G6PC gene to the patients as a means of extending over all gene mutations. For this to be effectively done, the G6PC gene must be implanted into the target cells with a single vector and in a single instance with safe integration. Due to the lower gene transfer efficiency for in-vivo treatments of non-viral vectors, viral vectors were chosen instead. [8] [9] [10] Through choosing the viral vector taking into account factors such as the lower potential to cause inflammation, the higher gene transposition [11] and gene integration capacity [8] [12], wider gene tropism range and the potential for the case proteins to be changeable [13], lentiviral vectors were chosen.

Although lentiviral vectors are designed to be safer with each generation [8] [14] they still carry the potential to activate a protooncogene by being infused in a random part of the genome through the integrase enzyme they contain [15] [16] . It was decided to transfer genes through 3rd generation [17] integrase defective lentivirus (IDLV) [18] to minimize the possible oncogenicity. However, having a defective integrase function will also mean that the gene transfer will be left episomal and will lose its effectiveness after a certain amount of time in replicating cellular groups, also in the targeted hepatocyte cells.[19]

To overcome this issue and to also integrate the G6PC gene to a specific target in the genome, the CRISPR/Cas9 system was added to the integrase defective lentiviral vector. [20]

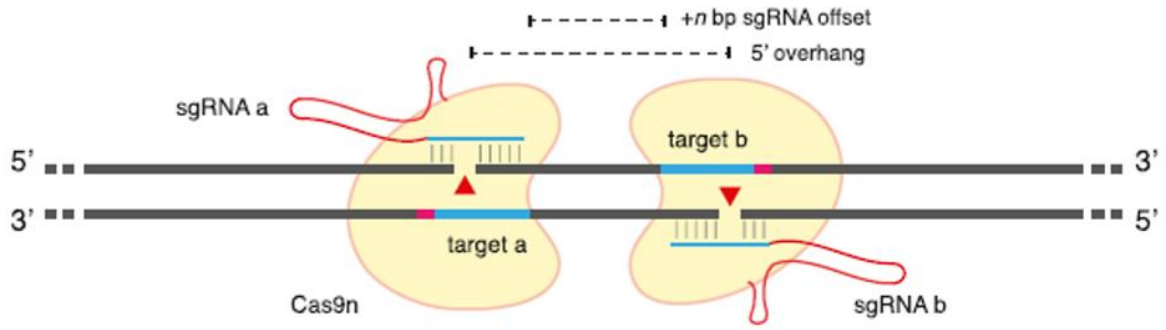
CRISPR/dCas9-FokI

The CRISPR method is a gene design system functioning through the attachment of a guide RNA (gRNA) complementary to the target DNA sequence, which then is recognized by the Cas9 enzyme to create a double strand incision. Following the double strand incision, one of two DNA repair mechanisms, HDR (Homology Directed Repair) and NHEJ (Nonhomology End Joining) come into effect for the repair. While the NHEJ repair mechanism has a high risk of creating an indel, the HDR repair mechanism has a high rate of accuracy in repair. [21]

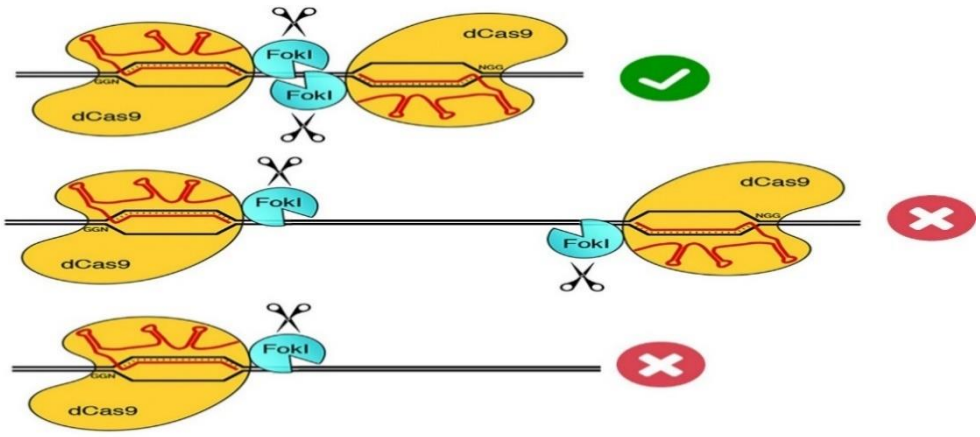
The PAM sequence, which is usually a 2-6 bp long specific sequence located in any one of the ends of the sequence targeted by the gRNA, is necessary for the CRISPR system to function. This sequence shows variation depending on the protein used in the CRISPR system.

Studies done on the CRISPR/Cas9 system since its first use have shown that it contains many disadvantages despite its advantages. Of these disadvantages, the most important are off target mutations, which are the binding of the gRNA to sites very similar to the target sequences and the Cas9 protein creating incisions in undesired sections of the genome. [22] Different CRISPR/Cas9 sought out to overcome the issue. It was found that CRISPR/Cas9 systems in which two gRNAs are used in combination have much lower off target mutation rates. [23]

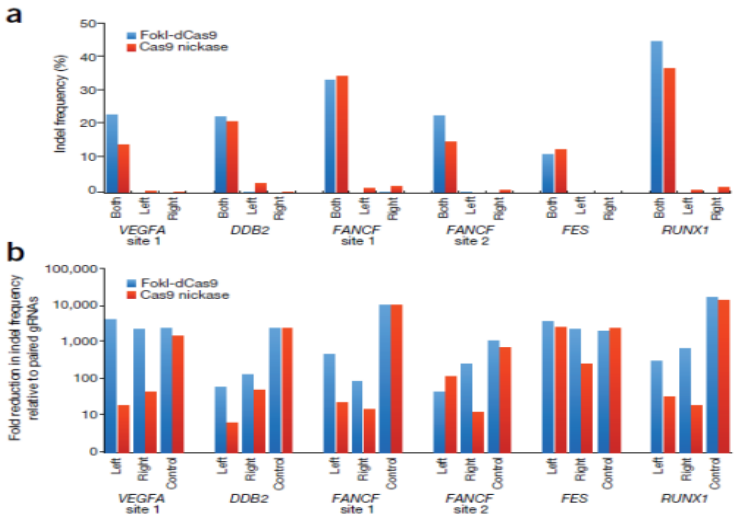
The first of such systems, the CRISPR/Cas9-nickase system, has a single one of the catalytic subunits of the Cas9 enzyme to create the Cas9-nickase enzyme that has the ability to crease an incision in a single strand. For a double strand incision to occur, two Cas9-nickase enzymes have to come across simultaneously. If the Cas9-nickase enzyme creates an incision in a single strange, the BER (Base Excision Repair) mechanism can repair the DNA. [24]



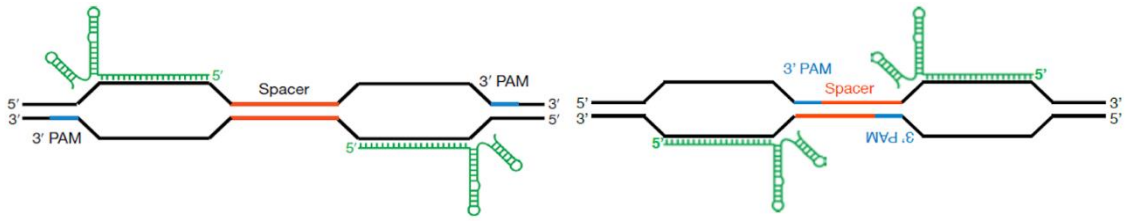
The second system, CRISPR/dCas9-FokI, has a Cas9 enzyme mutated in one of the catalytic subunits to create the dCas9 enzyme, which does not have the ability to create nicks on the genome, to then be merged with a monomer of the FokI enzyme. In this system, two different gRNAs are designed to target two nearby opposing sequences in the DNA double helix. dCas9 binds to the target gene sequences through the gRNAs and the FokI enzyme monomers come in contact in opposing strand to form a dimer and create an incision in the genome. If one of the gRNA were to bind to a different part of the genome, the FokI enzyme would not dimerize to create the incision. [25] Thus, off target mutations are significantly reduced.



In a study comparing the effectiveness and off-target mutation rates of the Cas9-nickase and dCas9-FokI systems, despite the effectiveness rates being found to be similar, the dCas9-FokI system has been found to create up to four times less off-target mutations in contrast to the Cas9-nickase system. [23] This was the reason for the use of dCas9-FokI in this study.



The dCas9-FokI system has certain specific qualities. The localization of the PAM sequences where the two different gRNA bind heavily alter the effectiveness of the system. Studies have shown that PAM-out systems, where the PAM sequences are on the outer sites of the gRNA, function more effectively than PAM-in systems. [26]



PAM-out PAM-in

Furthermore, optimum effectiveness was observed when the distance in between the target sites of the two gRNA (spacer) was 13-29 bp long. [23] [27]

The gRNA were designed especially taking into account these two factors

AAVS1 Safe Harbor/G6PC Gene Site

The region in which the functional G6PC gene will be integrated to the genome with the CRISPR dCas9-FokI system is crucial, as the gene may have unforeseen interactions with the genome in the integration site. This may reduce the therapeutic effect through reducing transgenin expression, as well as affecting the neighboring endogen genes to the integration site. The system may also cause oncogene activations or the suppression of tumor suppressor genes, leading to cancer formation. Studies done on retroviruses have shown that randomly integrated transgenes to the genomes of hematopoietic stem cells can cause preleukemia, myelodysplastic syndrome or acute leukemia. [28]

To prevent mentioned risks, genomic safe harbor regions were designated according to the literature and selected with the “Primum non nocere” principle in mind. The human genome is considered to have three safe harbor regions, which are the

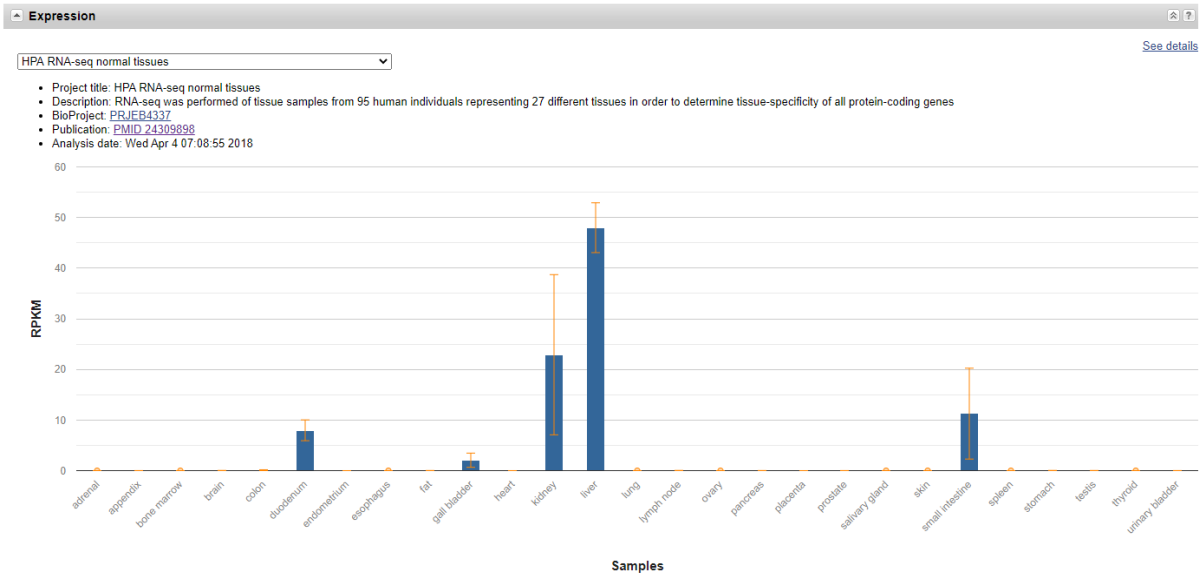
AAVS1, CCR5 and human ROSA26 regions. Many more regions aside from those mentioned have been found to be potentially safe. [29] The target selected in this was the AAVS1 region considering the on-target and off-target scores of our gRNA.

Considering the effects of gene regulation, it was also thought that the expression of the gene would be more natural and controlled with the effect of physiological transcription factors if the G6PC locus was to be targeted [30]

As a result of these considerations, the integration targets for the G6PC gene were designated as the AAVS1 safe harbor site and the G6PC gene site.

Liver Spesific Promoters

The G6PC enzyme is synthesized at the liver in the highest degree and in slim amounts in intestinal and kidney cells. [31] [32]



The viruses transfused to the system through circulation in the systemic treatment model will be able to reach tissues where the G6PC enzyme is not normally synthesized and bear the possibility for the synthesis to occur in those tissues. Such an example would be the skeletal muscles of the body, which could cause metabolic disorders. To prevent such occurrences and to only create a treatment model specific to liver tissue, the promoters for the dCas9-FokI system and the G6PC enzyme were required to be specifically designed. Such specific promoters were assigned from the literatüre. [33] [34] (hAAT, TBG, Albumin, G6PC promoter)

Despite the off-target mutation risk with the dCas9-FokI system being significantly lower in respect to other Cas9 systems, the continuous functioning of the system would pose a risk for mutations to occur. Hence, the liver tissue specific promoters were designed as doxycycline specific inducible promoters taking into consideration the side effects of tetracyclines in children, enabling the system to be controlled and to function according to the tetracycline administered. [35]

Tissue Spesific miRNA Target Sites

miRNA target sites were used as a secondary control mechanism to prevent potential non-target tissue synthesis of G6PC.

miRNAs are non-coding small RNA molecules, approximately 20-24 bp long, responsible in RNA silencing and gene regulation. miRNAs function through binding to the complementary regions in the mRNA sequences. [36] Studies have shown that different cell lines and tissue types have different miRNA groups. [37] These miRNA have been designated for each tissue type and used in various gene therapy research to limit target protein expression.[38] The liver specific gene therapy model has also been shown to create immune tolerance to reduce the immune response that may occur against the transgene through T-regulatory cells.[39] [40]

To make the functioning system more tissue specific and less immune responsive on the target tissue, miRNA target sites specific to muscle (miRNA 206) **[41],** hematopoietic (miRNA 142)**[42]** and endothelial cells (miRNA 126) **[43]** were placed at the end the dCas9-FokI gene sequence to limit G6PC production in these three tissues. Two positive selection miRNA target sites were also considered aside from the proven negative selection sites, the first being the liver specific miRNA 122 target sites placed around the gRNA to merge with their respective miRNA in liver cells to release the gRNA. [44]

The second positive selection site designated was the L7Ae kink-turn system. The L7Ae protein produced within the cell binds to the k-turn added at the start of the desired miRNA sequence to suppress protein synthesis. [45] Through the L7Ae protein, containing the miRNA 122 target binding site at its side, dCas9-FokI production is inhibited through binding at the k-turn located on the side of dCas9-FokI. As liver cells contain high amounts of miRNA 122, the production of L7Ae protein is suppressed and dCas9-FokI goes through disinhibition. Hence, the dCas9-FokI system will only be activated in liver cells that contain miRNA 122.

G6PC+

The literature has demonstrated that changes in the G6Pase-α isoenzyme (Ser-298 → Cys-298) found in dogs, mice, rats and certain primates increases G6PC enzyme activity.[46] Hence, it is expected that the use of the mentioned isoform instead of the WT G6PC gene will cause an increase in enzyme activity more than twofold

Liver Spesific Lentiviral Envelopes

To increase the specificity to the target tissue, reduce the rate of cytotoxicity, achieve a lower rate of serum inactivation and immune response for the lentiviral vector that will transmit the G6PC enzyme, a change on the lentivirus coat proteins was foreseen. This was designed through the RRV (E1, E2) [47] [48] and Baculovirus (GP64) [47] [49] [50] [51] proteins, which are more specific to liver cells compared to other options.

	Entry to Liver Cell	Other Non-hepatocytic cells It entered	Advantages	Disadvantages
RRV E1 E2 protein (most likely heparin sulphate and integrins*) [47] [48]	20 times higher entry to liver cells in contrast to VSV-G coated lentivirus. 100 times more entry to liver cells in contrast to other cells	Kupffer cells, neuroglial cells, Fibroblasts, Very low rates of skeletal muscle, respiratory tract	Lower cytotoxicity Lower serum inactivation, Lower immune response More resistant to centrifuging	There is some immune response, The protein that the virus binds is not clear
Baculovirus GP64 protein (Heparan sulphate, phospholipids) [47] [49] [50] [51]	Similar levels of entry to liver cells with to VSV-G coated lentivirus 100 times less entry to dendritic cells	Fibroblasts, epithelial cells, To other cells in lower rates	Lower cytotoxicity More resistant to centrifuging Almost no hematopoietic cell transduction Lower immune response Half the amount of serum inactivation in contrast to VSV-G coated lentivirus	There is some serum inactivation
VSV VSV-G protein [47] [48] [49] [50] [51]	High rate of entry to many cells including liver cells	Many cells, neurons	More standard usage	Less specificity to the liver, Higher rate of serum inactivation and immune response

Route of Administration

Finally, we investigated the way in which lentiviral therapy can be given to the body. We concluded that using the IM injection route is disadvantageous because the vector given in very high doses in intramuscular injection creates a high antibody response, develops local lymphocytic infiltrates, the effect area is only in a limited area around the needle and requires too many injections.[41] [52]

IV administration on the other hand requires much smaller doses to have much greater outreach. The lower doses will allow for less immune response.

The application of the lentiviral through the hepatic artery or the portal vein was deemed a more direct path for the treatment to reach the target organ, which is the liver. The portal vein has a bigger percentage in the perfusion of the liver and is more suitable for this role. However, invasive radiological procedures using catheters bear certain risks. [53]

Hence, administration through the peripheral veins and the portal vein will be tried and compared to find the more effective method.

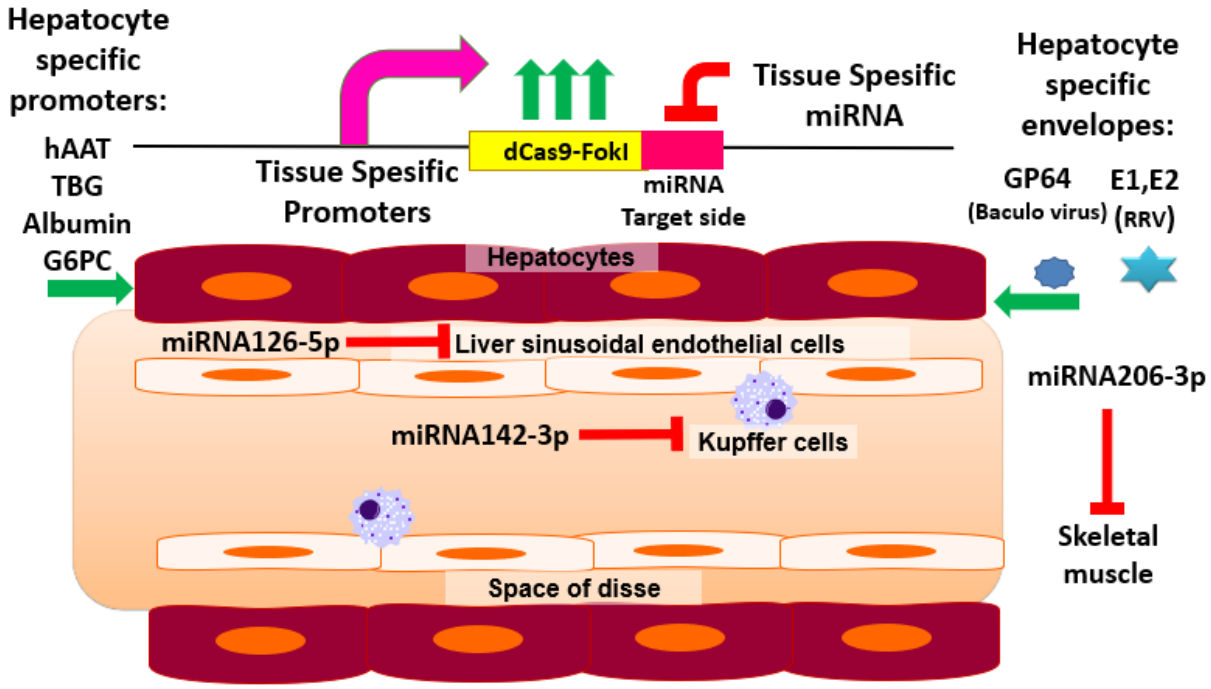
GSD type IA gene transfer studies have shown that tumor proliferation is prevented in mutated cells where G6PC enzyme activity is ≥ %1. [54]

When the G6PC enzyme activity is ≥ %3, it has been shown that the cells can manage normal glucose homeostasis. [46] Furthermore, proliferating hepatocytes also carry the ability to replace damaged tissue. As the mutated cells will have reduced proliferative capacity and cellular lifespan in contrast to repaired cells, the number of cured cells will proliferate with time to replace and constitute the majority of liver tissue, even if the therapy could reach the genomes of a few number of cells in the tissue. [55]

To sum up, with the **“Primum non nocere”** principle in mind;

- To minimize the vector caused side effects and have more efficient usage by requiring only one time administration, lentivirus was chosen as the vector.
- To prevent the natural integration of lentivirus to the target genome that could cause mutations and oncogenicity, integrase defective lentivirus was selected.
- To prevent the transferred genetic material from being left episomal to lose effectiveness over time, CRISPR/Cas9 system was chosen to target a specific part of the genome.
- As the CRISPR/Cas9 system has high off target mutation rates, the dCas9-FokI system was used to minimize the mutation.
- Two target sites for gene transfer were chosen, first being the natural site of the G6PC gene for natural enzyme regulation and the second the safe harbor region AAVS1 to minimize mutation risk.

- miRNA target sites and lentiviral coat proteins were specifically chosen and designed to deem the gene transfer specific to liver tissue.
- To impede the potential risks of the continuous functioning of the system, a tetracycline dependent and liver specific promoter was elected to act as an on and off switch.
- A more active isoform of the G6PC gene was taken to increase therapeutic potential.
- It was decided that the most practical and effective method of administration would be IV. Despite application directly through the portal vein being more a more direct treatment to the target organ, it was decided to compare administration through the peripheral veins and the portal vein for the optimal method, as invasive radiology methods have their own risks..



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