RARE DISEASE CHALLENGE
RaDiChal'21

FINAL PRELIMINARY REPORT

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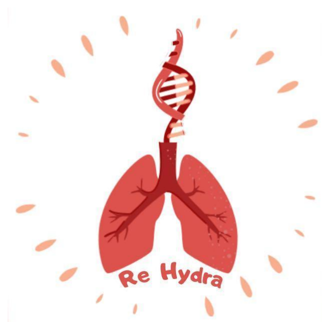

GENE EDITING, REHYDRATION OF DEHYDRATED MUCUS PHENOTYPE IN CYSTIC FIBROSIS, TARGETING PROGENITOR EPITHELIAL CELLS WITH MODIFIED MRNA TECHNOLOGIES AND CRISPR PRIME EDITING

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1. *Cystic Fibrosis Description*

Cystic Fibrosis (CF) is one of the rare diseases with autosomal recessive inheritance. CF is caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane Regulatory Protein (CFTR), which has a chloride (Cl) channel function in the cell membrane. The CFTR gene is located in the q22-31 region of the 7th chromosome[1] with a total length of 230 kb, it contains 27 exons, its mRNA is 6128 nucleotides, and its open reading frame (ORF) is considered to be 4.4kb. The protein structure of CFTR includes 1480 amino acids with a protein weight of 168kDa.[2,3]
CFTR is also known with a name of 'cAMP dependent chloride channel' which has a catalytic activity as follows;
ATP + H(2)O + closed Cl(-) channel= ADP + phosphate+ open Cl(-) channel[4].

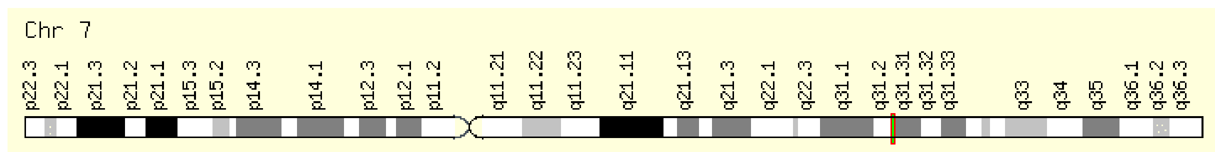

Figure 1: The CFTR gene chromosome location(7q31.2).

CFTR protein is expressed in many cell types found in the airway epithelium, submucosal glands, pancreas, liver, sweat glands, and reproductive organs. In the case of a genetic defect that causes CF, ion transport from epithelial surfaces gets disrupted, and the ability of epithelial cells to secrete chloride and absorb sodium in response to agonists acting on c-AMP decreases. The failure of chloride and fluid secretions in the respiratory epithelium as a result of the defect in ion transport leads to dehydration of the mucus, impaired mucociliary clearance, and eventually the development of lung disorders seen in patients with CF. Another fact is the relative darkening of secretions in the pancreas and liver, which results in on

congestion in the ducts. Moreover, the electrolyte concentration in sweat increases due to the disruption of the transductal reabsorption of chloride and sodium in sweat glands,[5].

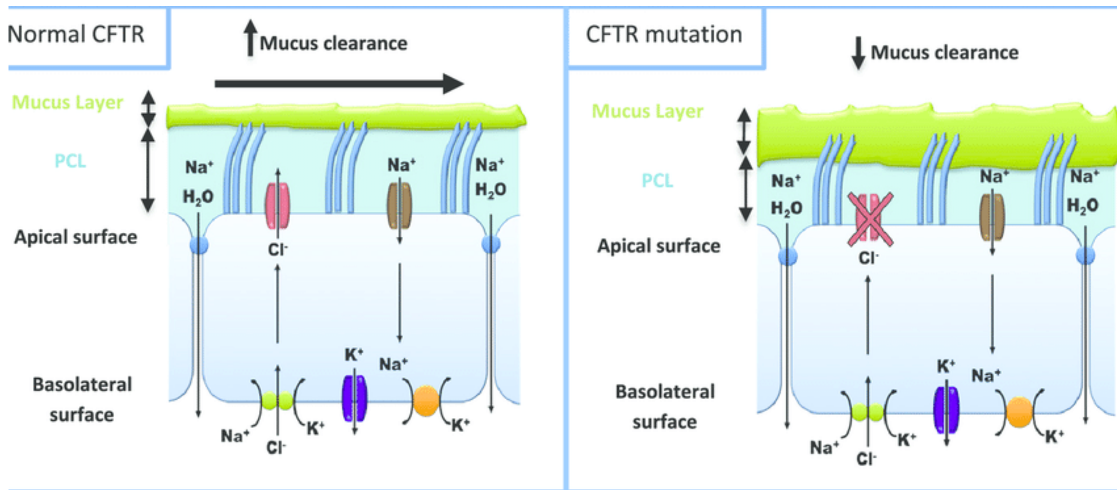

Figure 2: Normal CFTR channel and mutant CFTR channel that causes the disease, schematic view[7].

Almost 1,700 CFTR sequence mutations and variations have been identified so far. The most common mutation worldwide, in the CFTR gene, is ΔF508, the deletion of the 508th codon, which encodes the amino acid phenylalanine, of CTT nucleotides.[6] Thus, a folding defect occurs in the CFTR protein and a rapid degradation occurs in the endoplasmic reticulum (ER) process[8]. To mention the most common CF genotype which is homozygous ΔF508/ΔF508 and this mutation accounts for 70% of the allele frequency.

Table 1 The ten most common CF disease alleles.

Allele Frequency[a]	Mutation	DNA Change	Class	CFTR Modulator
70%	F508del	delCTT	deletion	VX-661/ VX-770 VX-809/ VX-770
2.5%	G542X	G > T	transversion	
2.1%	G551D	G > A	transition	VX-770
1.5%	N1303K	C > G	transversion	
1.3%	R117H	G > A	transition	VX-770
1.2%	W1282X	G > A	transition	
0.93%	R553X	C > T	transition	
0.93%	621+1G > T	G > T	transversion	
0.86%	1717-1G > A	G > A	transition	
0.82%	3849 + 10kbC > T	C > T	transition	VX-661/ VX-770

[a] Data from the CFTR2 project.[62]

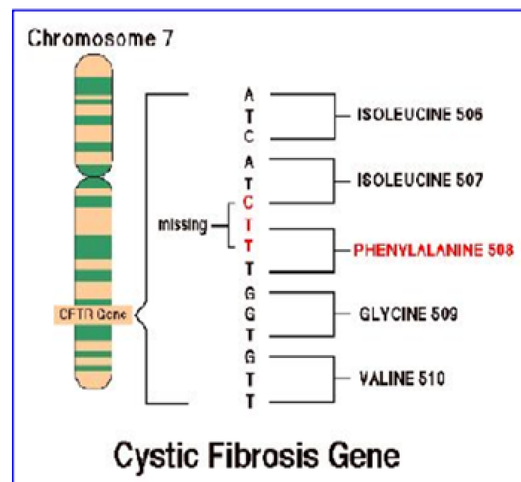

Figure 3: The most fraquent 10 CF mutations.

Figure 4: CFTR gene exon 11 CTT region.

Mutations in the CFTR gene can be classified into 6 classes according to their molecular mechanisms and consequences for different aspects of CFTR biogenesis, metabolism, and function. Class I mutations produce an unstable mRNA that results in a protein synthesis defect. Class II mutations affect protein maturation. Class III and IV are classified as channel mutations, class III mutations alter the channel regulation. , class IV mutations affect chloride conductance. Class V mutations reduce the level of normally functioning CFTR at the apical membrane. Finally, class VI mutations decrease the stability of CFTR present or affecting the regulation of other channels and limit its function[8].

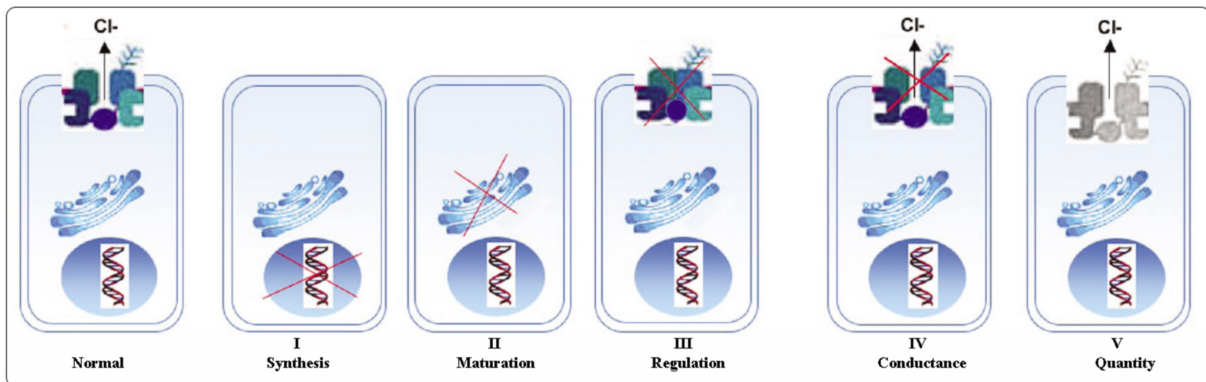

Figure 5: CF mutation types[8].

Class II mutations, which we will particularly focus on in a part of our project, are characterized by misprocessing of CFTR resulting in the absence of functional protein in the plasma membrane. A prime example of a class II mutation is F508del located in the nucleotide-binding domain (NBD1) and produces a misfolded polypeptide that is recognized by the ER quality control mechanism, tagged with ubiquitin, leading to degradation by the proteasome[9].

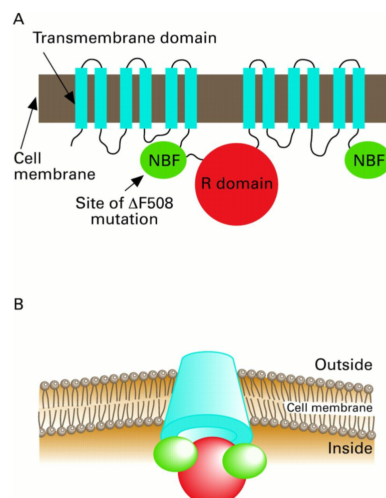

Figure 6:ΔF508 mutation of CFTR that affects first nucleotide-binding domain (NBF).

Cystic Fibrosis is one of the most common fatal diseases among rare diseases. In studies conducted throughout Turkey, its frequency has been reported to be one in 3000 births.

However, the incidence of the most common ΔF508 mutation in the CFTR gene is much lower in Turkey (20-25%). In this context, mutation-specific genetic treatment methods continue to remain in the background.

To increase the CF patient's life expectancy and living standards, which has no definitive treatment yet, many drugs have been tried with the development of drug technologies. Recently, modulator drugs have been developed for CF patients and have become a treatment option for patients. However, modulator drugs cannot provide a complete cure for CF patients and can only be used by patients with certain types of mutations. The fact that modulator drugs offer a limited treatment opportunity for CF and that they only appeal to certain patients, also, their high costs lead the patients to have difficulty in accessing modulator drug treatments. In addition, the necessity of continuous use of modulator drugs limits the access of these drugs, which already have high costs, by patients[1]. For these reasons, the development of genetic treatments for CF comes to the fore. The main aim of our project is to develop a genetic therapy project for the treatment of CF, trying to solve the pathological problems of the rare disease, Cystic Fibrosis.

2. *Who is Re-Hydra, Aim and Therapy Methods*

The Re-Hydra team consists of five different university students who aim to produce a genetic therapy project for one of the rare diseases, Cystic Fibrosis.

The Re-Hydra team aims to develop a genetic therapy project for Cystic Fibrosis, to rehydrate the -dehydrated- mucus that has lost its fluidity by making it - re-fluidized. Projects, the goal is to both repair and tolerate different types of mutations in the CFTR gene that cause Cystic Fibrosis. Constitutively is aimed to normalize the control of ion-water secretion and absorption of the CFTR gene in epithelial cells leading to the restoration of chloride transport in the cell membrane. The CFTR gene is transcribed at low levels in the body, and the mature protein product may be stable for long periods of time with a half-life of >15 hours after reaching the plasma membrane. Researches have been made considering this phenomenon, accordıngly, we have found evidence that restoring 10% of CFTR mRNA or 10-35% of protein function wıll be sufficient to cure the disease, and our project was designed on the basis of these criteria.
Literature surveys have been made for our project, and two different designs, A and B projects, have been developed. Our designs will be used with an in vivo-non-viral vector and it is decreed to confirm the treatment developed with in vitro techniques. Finally, studies have been carried out for the best animal model which are the next stages of our project, to be able to mimic the most appropriate cystic fibrosis phenotype for the preclinical and clinical trials, and for the optimum feasibility level of the project.

3. *Project A*

As the Re-Hydra team, our Cystic Fibrosis genetic treatment approach is CRISPR-based as Project A. Nowadays, CRISPR-based genetic therapies have become very popular, with the CRISPR-Cas system winning the Nobel Prize. In our Cystic Fibrosis genetic therapy project A, it is aimed to use the Prime Editing-PE2 system, which is an improved version of the CRISPR method, in the lung due to its lethality in the CF phenotype. The target cells of our PE2 system are lung progenitor cells. In this way, we will restore CFTR activity by an insertion (CTT) of the Cystic Fibrosis ΔF508del mutation. Prime Editing is a more sensitive, more efficient, and versatile technology that allows genetic editing on human cells. This technology makes it possible to treat approximately 89% of diseases caused by genetic mutations[10,11].

The main reason for choosing the Prime Editing (PE2) system is that the Cas9-H840A/pegRNA complex is modified to bind to the desired target site and cut only one strand of DNA from the desired portion. Thus, it creates a 3 bp nick upstream of the abundant PAM-NGG region in the genome, which is determined as the target PAM sequence. In this way, after the nick in the targeted region, PBS anneals and RT is reverse transcribed. This results in an intermediate with a 3' and 5' ssDNA flap containing the first embodiment. When this regulated flap undergoes ligation, a heterodimer gets formed, followed by fusion of the CTT-edit.

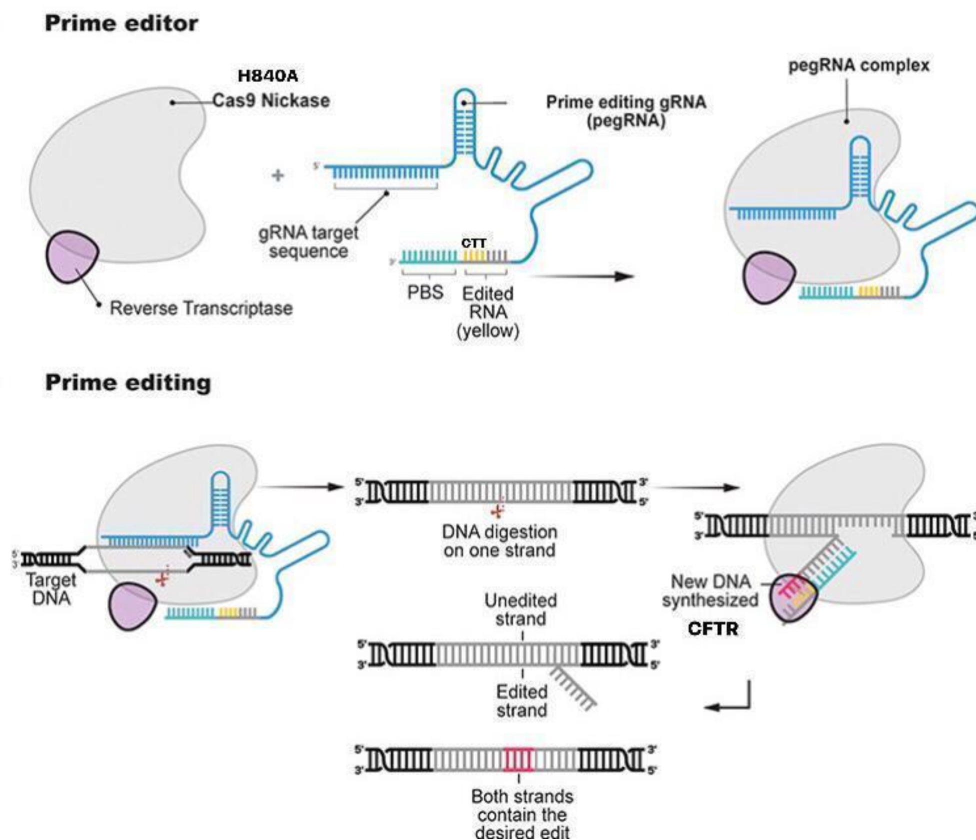

Figure 7: Genetic editing with Prime Editing Cas9 Nickase H840A[12]. The figure is edited for Cystic Fibrosis by ReHydra.

Using this method, our optimized Prime Editing components will be transferred to the specific expression plasmid (which is designed by ReHydra, modifying the pegRNA expression plasmid) for genome editing in individuals with Cystic Fibrosis. In addition to our optimized pegRNA, we have designed more than one pegRNA for comparison. The Cas9-H840A enzyme was chosen for its success in insertions and contains an activity-optimized M-MLV (Murine leukemia virus) reverse transcriptase fused to it. Also, our plasmid contains a tissue-specific (predominantly in the epithelium of internal organs (it shows a distribution pattern in lung, liver, kidney, and intestines, this distribution matches exactly with the CF phenotype)) promoter and enhancer by that way the expression can be compatible with CFTR. Apart from pegRNA suitable for genetic therapy it also contains the necessary marker-selection genes.

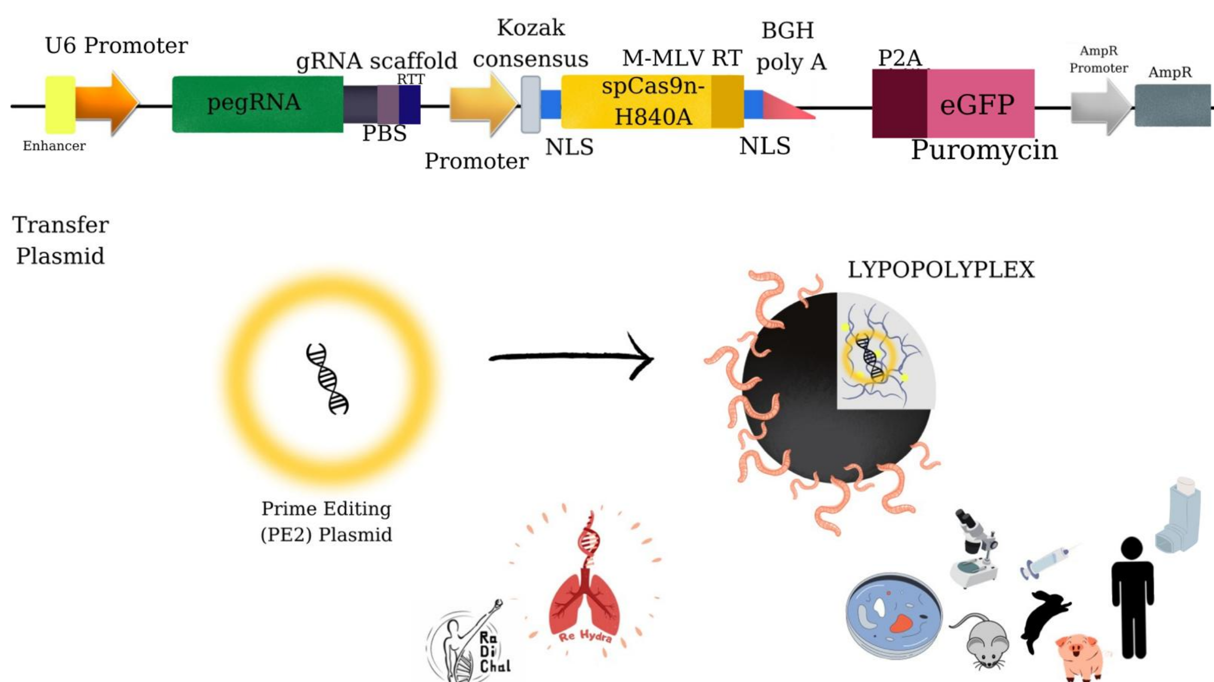

Figure 8: Schematic representation of the designed plasmid and its delivery with an LPP vector.

The non-viral vector was chosen considering the size of the Prime Editing system and its safety profiles. Combining the advantages of both polyplexes and lipoplexes in non-viral vector selection, Lipopolyplexes (LPPs) were chosen for our project. LPP is a triple nanocomplex of cationic liposome, polycation, and nucleic acid, and there is no theoretical packaging limitation.

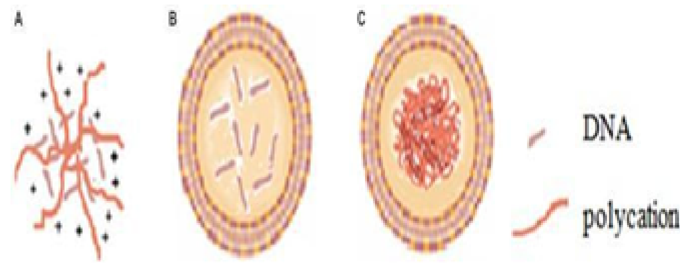

Figure 9: A.Polyplex, B.Lipoplex ve C. Lipopolyplex[13].

In this way, we have avoided the problem of expression plasmid packaging limit for a large genome-editing system such as Prime Editing. The non-viral vector, after being designed with polycation compounds which have lung tissue tropism and cationic lipid composition specific to airway cells. Also, LPP will contain a mucoadhesive compound in its outer layer so that it can easily pass through the mucus layer, which is seen as a transfer barrier in CF treatment and may affect vector distribution. Finally, in order to overcome the transmission barriers, it is planned to use mucolytic agents before the transmission, afterwards, the LPP will be delivered by inhaler or endotracheal methods. In addition, in case of a different organ is desired to be targeted, the non-viral vector can be applied to the same system by changing the tissue-specific compounds.

Combining all of these, firstly our project will be applied to our target mutation-specific in vitro cell line, then in pre-clinical experiments in animal models that can reflect the CF phenotype, especially lung phenotype, and most recently in clinical trials. In this way, the Prime Editing (PE2) method will be applied to the CTT in exon 11 of the CFTR gene and will lead to correction of the deletion (HomF508del and HetF508del). Accordingly, it will be ensured that the CFTR protein is expressed in the correct folded manner and preventing it from going into the ERAD complex.

Finally, confirmation methods are planned at experimental stages, as the recovery of CFTR function will be causing changes, at certain stages determination will be made; at the gene level, at the protein level, and finally at the molecular level because of the Cl flow of the membrane potential will be changed after the correction. Additionally, dosage determination will be made with trials in the pre-clinical stages.

4. Project B

ReHydras' major project is the B which is based on literature reviews, in order to correct all mutational defects that cause Cystic Fibrosis disease we aim to transfer the modified CFTR-mRNA of the CFTR gene to the lung epithelial progenitor cells. In this way, it is aimed to produce CFTR protein effectively as a result of translation of CFTR mRNA in the cytoplasm, without the need for a CFTR DNA to enter the nucleus of the cell. As the delivery method, the LPP system has been chosen due to its advantages such as transient, high tissue tropism, no packaging limit, non-integration into the genome, non-toxicity, and high

reliability among non-viral vectors. Furthermore, the LPP system contains chemical compounds to protect the mRNA from degradation during transport.

The production of in vitro transcribed mRNA (IVT mRNA) is easier than the production of DNA and can be standardized while maintaining its reproducibility. This approach has become even more popular in recent years with the improvement of delivery technologies and mRNA vaccine systems in the COVID19 pandemic. The delivery of a therapeutic nucleic acid (mRNA) is an intellectually satisfying solution for an inherited single gene defect such as CF, with the possibility to correct many aspects of a complex pathology. is the concept. Besides, a potential RNA therapy for CF could be tested in an early-stage clinical trial to determine safety[14].

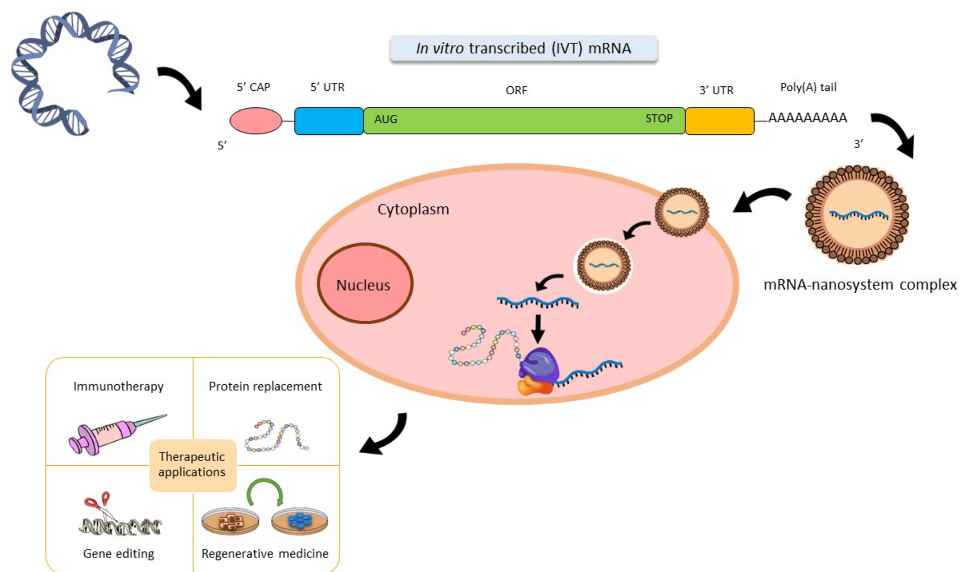

Figure 10: Schematic view of IVTmRNA therapy.

During the development phase of the project, new mRNA technologies used in vaccines have been examined, particularly mRNA vaccine systems of companies such as Moderna (mRNA-1273), Biontech (BNT162b2), which everyone is familiar o companies due to the pandemic. In contrast, CFTR mRNA studies that have not been disclosed yet, have been examined, but since the content of projects are not stated, Covid-19 mRNA modification systems were evaluated by us, screeing the pros and cons of the modification systems used. There are multiple mRNA vaccines for Covid-19, but only two (Biontech, Moderna) of them have been ultimately successful. The main reason for this is the modifications they contain; 5'CAP modifications, 5' and 3'UTR optimization, codon optimization, polyA, and termination systems are some of them. Although the two vaccine systems are considered basically similar, they actually follow very different modification paths.

In order to have a long-lasting mRNA therapy other than the usual short period of time therapy such as one or two weeks, and to use minimal repeated doses, COVID-19 vaccines stability-enhancing modifications have been examined for our CFTR-mRNA. Unfortunately, exogenous mRNA can be detected and destroyed by serum nucleases and can trigger an immune response upon cellular entry. To avoid that current strategies use chemically modified mRNA containing modified nucleosides such as pseudouridine. These chemical modifications help effectively knock down the innate immune response and provide enhanced stability. Based on the literature reviews conducted by our team, for these modifications, optimal modifications for CFTR mRNA have been determined.

Based on literature searches, the mRNA we modified includes codon-optimizations—the CFTR open reading frame (ORF), as well as the 5' and 3' untranslated regions (UTR), are optimized for translation efficiency and intracellular stability, an optimized Kozak sequence and termination codon, modified 5'CAP, the appropriate length PolyA tail, and contains the necessary nucleoside modifications to avoid activation of innate immunity. Innate immunity activation can inhibit CFTR protein production by affecting translation efficiency, while nucleoside modifications increase the stability of RNA by preventing hydrolysis by phosphodiesterase enzymes, and also increase the secondary structure stability of RNA. For CFTR mRNA therapy to be clinically relevant, long-lasting CFTR protein expression after mRNA delivery is required. In this context, improvements have been made in mRNA stability. To point out, N1-methylpseudouridine, which has achieved outstanding success during the pandemic process, will be used in our project.

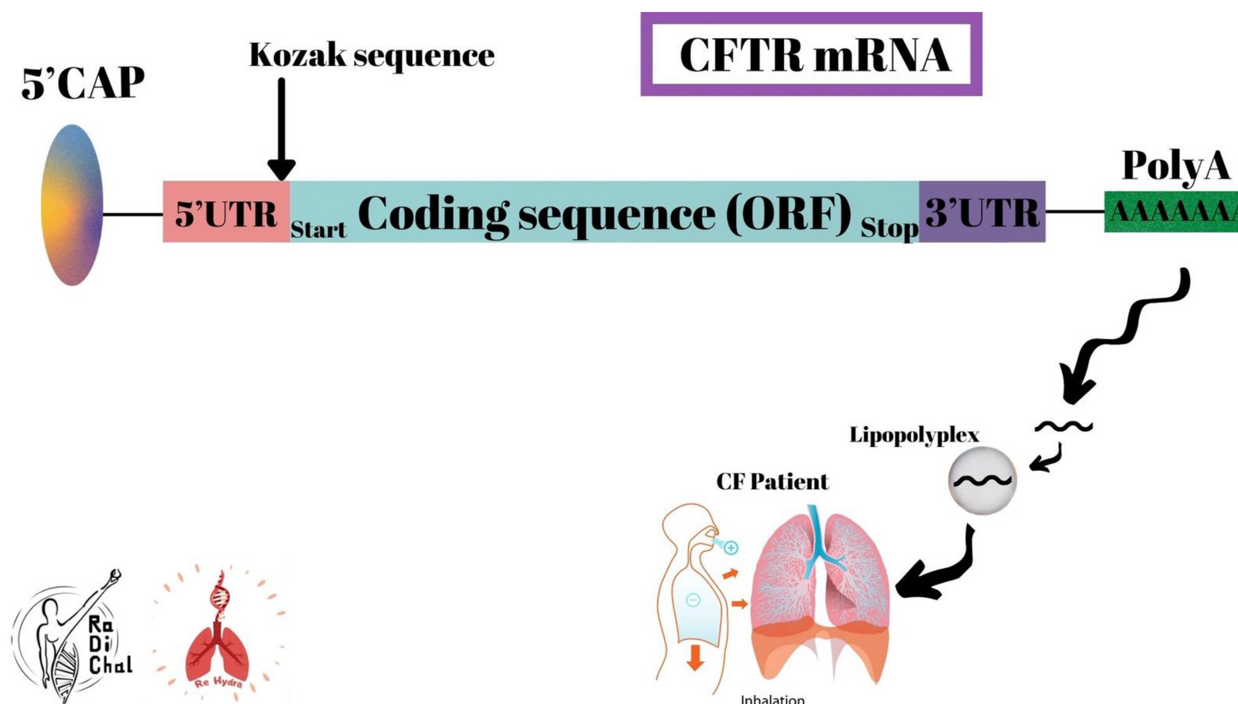

Figure 11: Schematic of the CFTR-mRNA approach by the ReHydra team.

In addition, the lung organ that results with the most lethality in CF has been chosen as the main target, the target cells of the treatment were selected based on their longevity compared to other lung cells. For this reason lung epithelial progenitor cells have been chosen. Although vector optimizations are done tropically, in the case of necessity of targeting a different tissue (eg, CF gastrointestinal tract) can be achieved by changing components of the LPP system.

The modified CFTR mRNA will be transferred to the LPP system after being synthesized and modified as IVT, then tested in the cystic fibrosis cell line in in-vitro. An immortal cell line that can show all the ion transport features of cystic fibrosis, such as defective cAMP-dependent chloride transport and impaired calcium-dependent chloride transport, has been chosen as the cell line[9]. In the animal experiment phases of preclinical studies, it is considered valid to choose an animal model of CF that can ideally characterize the lung mucus phenotype and bacterial infections in order to accurately measure the efficacy of the treatment. For this reason, based on the literature surveys, 4 animal models are selected considering the laboratory conditions and the level of reflection of the lung CF phenotype most effectively; Rat, Ferret, Rabbit, and Pig. Another crucial aspect of the reflectivity of the CF phenotype is that barriers in the CF phenotype will definitely affect vector transmission. In order to overcome the barriers, the use of mucolytics in pre-clinical and clinical stages, before the inhaler or endotracheal delivery of the vector, is decided.

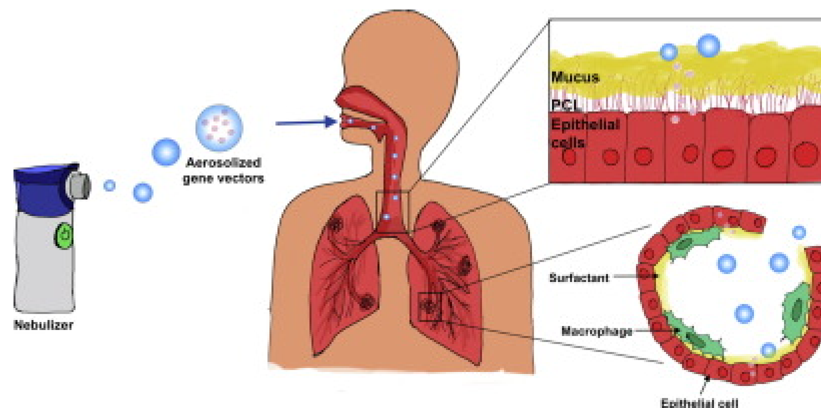

Figure 12: Schematic representation of CF inhaler-aerosol gene therapy [15].

In conclusion, confirmation methods are planned at experimental stages, as the recovery of CFTR function will be causing changes, at certain stages determination will be made; at the gene level, at the protein level, and finally at the molecular level because of the Cl flow of the membrane potential will be changed after the correction. Additionally, dosage determination will be made with trials in the pre-clinical stages. In addition, in our mRNA studies, mRNA-specific confirmation experiments such as mRNA half-life assay will be performed.

As mentioned earlier, the CFTR is a low-level expressed gene and the mature protein product can be stable for long periods of time with a half-life of >15 hours after reaching the plasma membrane. This should be considered firstly in any method that is considered to be applied. The capacity of the CFTR-mRNA transfer method to produce protein in a physiological way has been designed, and considering that LPP causes transient expression, it is predicted that the treatment life will be 6-9 months in low repeated doses thanks to its optimization with modifications. All data of the project will be statistically verified as significant using tests such as the Mann-Whitney U test, student-t-test.

5. *Conclusion*

CF, which is among the rare diseases, is a disease in which treatment options are limited due to difficulties such as insufficient information about the disease caused by the small number of affected individuals, few clinical trials, difficulty in drug development, and high costs of developed drugs. Although modulator drugs developed for CF offer a cure for the disease, they are not a comprehensive treatment option for all mutation types, for all patients, and are difficult to access with their high costs. Genetic therapy approaches, on the other hand, have the potential to offer a broader and more inclusive treatment for CF and hope to become a more accessible treatment with a single dose-one single-shot application.

As the Re-Hydra team, we have designed two genetic treatment projects, one based on the CRISPR-Cas9-PrimeEditing system and the other based on CFTR modified mRNA, in order to treat the pathogenesis of Cystic Fibrosis. In this direction; Our team aims to restore the CFTR function by making the most appropriate plasmid design, CFTR-mRNA modifications, optimization, and vector design with high tissue tropism so that the genetic treatments developed with maximum efficiency reach the lung progenitor cells determined as target cells and show effectiveness.

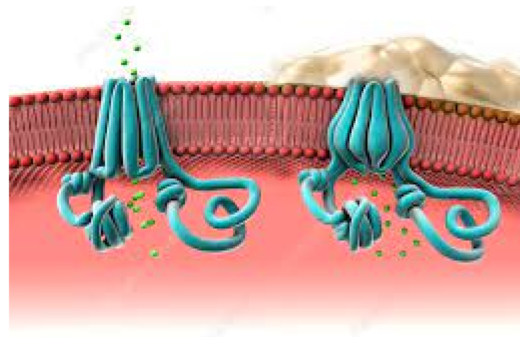

Figure 13: Normal CFTR and Abnormal CFTR[6].

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