RARE DISEASE CHALLENGE
RaDiChal'21

FINAL REPORT

TEAM NAME

FMF16E

TEAM MEMBERS

Ayşenur SAYGILI
Betül ÇAKMAK
Kübra MAÇ
Sena KIZILBOĞA

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TARGET DISEASES

FMF

Contents

1. Project Summary (Project Description)

FMF (familial Mediterranean fever) disease is an autosomal recessive disease caused by mutations in the MEFV gene located on the short arm of chromosome 16 (p13.3) of the human genome.

There are more than 300 single point mutations in the MEFV gene that have spread throughout the gene and have been identified to date. The MEFV gene consists of 10 exons and 781 amino acids and contains the Pyrin protein inflammatory codes. Mutations are mostly clustered in exons 2 and 10 of the MEFV gene. Pyrin protein affects the inflammatory mechanism. Since PNK1/2 proteins are unable to phosphorylate mutant Pyrine and then 14.3.3. As the proteins cannot bind to Pyrin, an inflammatory response occurs as a result of ASC and Pro-caspases binding to Pyrin.

Since the main affected structure in FMF disease is blood, it is expected that the treatment to be made over hematopoeitic stem cells will be a solution to the disease. This designed project is designed to be carried out ex vivo and then in vivo.

It is known that keeping the interference at the gene level to a minimum without interfering with the physiological promoter region of the gene and using HiFi-Cas9 in the CRISPR Knock-in method reduces the off-target mutation rate up to 20 times. The gRNAs to be used with HiFi-Cas9 were selected more than once, taking into account different off-target values. Reliability in transmission will be increased by combining it with the RNP method.

Gene tracking will be provided by placing the EGFP sequence at the end of the designed Donor DNA. The P2A sequence will be added for EGFP expression. 800 base homology arms will be added to the right and left parts. According to data from literature searches, it has been shown that adding these homology arms less than 500 base pairs reduces the effectiveness of knock-in much more in low-efficiency therapies such as knock-in. Extra stop codons will be added in front of the downstream Homology arm. These extra codons are additional countermeasures to the possible gene transcript.

In the electroporation method stage, the electroporation stage was strengthened by using the 4D Nucleofecker. At the proof of concept stage, stem cell differentiation and MEFV gene activity will be carefully taken and the cytokine levels of the selected cells will be examined and control tests will be performed. For in vivo clinical studies, humanized MEFV model mice are used to analyze certain symptoms and changes in gene level, and after providing control experiments, it is planned to switch to human trials. We think that if we treat the blood cells in the blood for FMF disease, we can solve the problem.

2. Problem/Issue:

FMF is an autoimmune disease and mutant pyrin produced in immune system cells triggers the inflammatory response. Since it is not a tissue-specific disease, our goal is to improve the treatment of this disease by correcting the immune system cells in the blood with gene therapy. Also, FMF is an autosomal recessive disease, so a change in a single allele of this gene will make gene therapy successful.[1]

2.1. A Problem at the Gene Level

• The MEFV gene in the human genome; 16. it is located on the short arm of the chromosome (p13.3). The MEFV gene consists of 10 exons and 781 amino acids and contains the codes for the Pyrin protein. Several mutations occur in the codalian MEFV gene of the pyrin protein. Mutations of the Pyrin gene 2, 3, 5 and 10. it is located in their exon (Figure 1). FMF consists of mutations in the MEFV gene.[2]

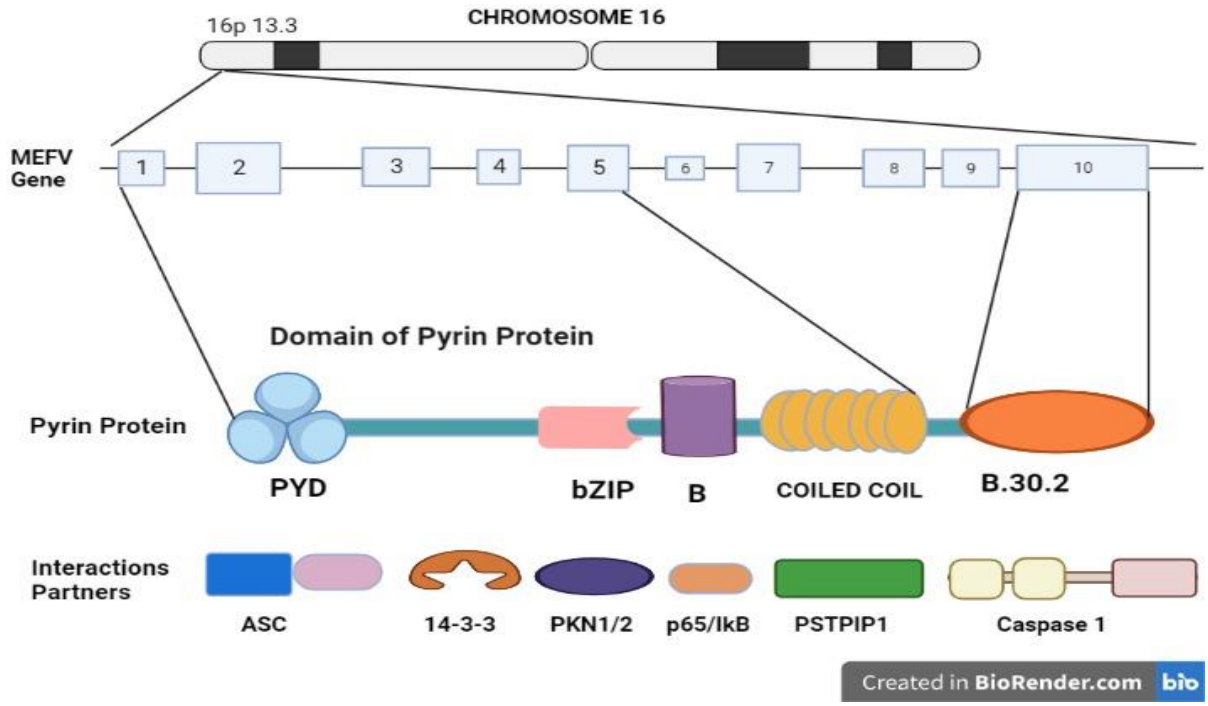


Figure 1: A show of the chromosome and the protein pirin, which are similar to FMF.

2.1.1. The Distribution Scale of the Problem at the Gene Level

The disease is inherited as an autosomal recessive trait. Hereditary diseases are determined by the combination of genes for a specific feature on the chromosomes from the father and mother. Etipyogenesis of mutations, the cause of the occurrence and development of a disease, is unknown.

FMF disease is common mainly in Jews, Spaniards, Greeks, Armenians, Arabs and Turks of Eastern European origin. There is a possibility that this disease, which is transmitted hereditarily, can also be observed in other people. The symptoms of lifelong FMF disease and the severity of these symptoms may vary from patient to patient. Although it is known as Mediterranean Fever, it is more common in the Inner Anatolia Region in our country as a result of studies. (Tablo 1).[3]

Tablo 1:The incidence of FMF mutations observed in regions of Turkey. n: percentage [4]

Study region	**Numbers of patients (pts)**	**M694V**	**V726A**	**M680I**	**E148Q**
Eastern Anatolia	453	36.5 (215)	14.09 (83)	3.9(23)	32.77 (193)
Eastern Anatolia	415	21.6 (180)	9.7 (81)	9.5(79)	19.1(159)
Southeastern	147	26(50)	13(20)	6.3(25)	30.7(59)
Southeastern	104	18.3(19)	8.6(9)	6.7(7)	30.8(32)
North East (Black Sea)	1620	42.8(-)	16.3(-)	14.1(-)	14.7(-)
Central Anatolia	2067	14.68(607)	4.78(197)	7.62(385)	5.15(228)
Central Anatolia (Ankara)	230	43(-)	*	12(-)	*
Central Anatolia	330	50(330)	9.7(64)	14.1(93)	1.36(9)
Central Anatolia	802	14.2(228)	5(87)	4(62)	4(71)
Western Anatolia	383	41.15(93)	7.08(16)	12.3(28)	20.3(46)
Aegean Region	1021	47.6(375)	12.95(102)	11.94(94)	16.75(132)
Mediterranean	1000	7.95(159)	1.85(37)	2.4(48)	8.85(177)
Overall Turkey	1090	51.4(1121)	8.1(188)	14.4(313)	*

*Frequencies not reported

In the table above, respectively, the most common mefv mutations in various ethnic groups of the Turks and the Armenians, M694V, M680I, V726A, E148Q; the Arabs, V726A, M680I, M694V, M694I, E148Q; in Ashkenazi Jews E148Q, V726A and Jews V726A, M694V, M680I and E148Q. Based on these data, 2 of the mutations. ,3. , 5. and 10th. Due to the fact that it is observed in exons, it is aimed to correct all mutations via the MEFV gene. With the method

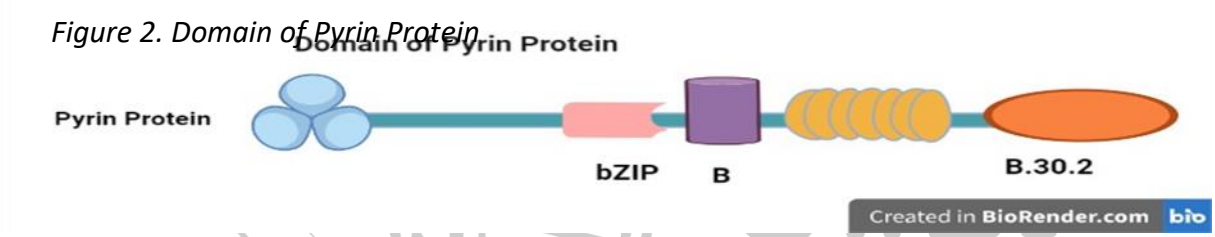
we aim to develop, it is expected that all heterozygous and homozygous mutations will be corrected.[4]

2.2. Protein-Level Problems

2.2.1. Problems with the Structure of the Protein

The pyrin protein has a unique structure. Pyrin is an inflammatory sensor that detects the inhibition of the activity of GTPase, a subfamily of RhoA. In october to a Pyrin Domain (PYD) at the N-terminal part of the pyrin protein, pyrin contains a B-box domain, followed by a helix-coil domain and a B30.2 domain. Most FMF-related mutations cluster in the C-terminal B30.2 domain of human Pyrin.[5]

Figure 2. Domain of Pyrin Protein



As a result of intramolecular interactions, it is also likely that B30.2 domain mutations control the phosphorylation of pyrin by inhibiting the binding of kinases to pyrin. Thus, it is shown that mutations in the B30.2 domain and other FMF-related mutations block phosphorylation sites from kinases such as PKN1 (figure 2), resulting in a lower threshold for the activation of the inflammatory of pyrin. [6]

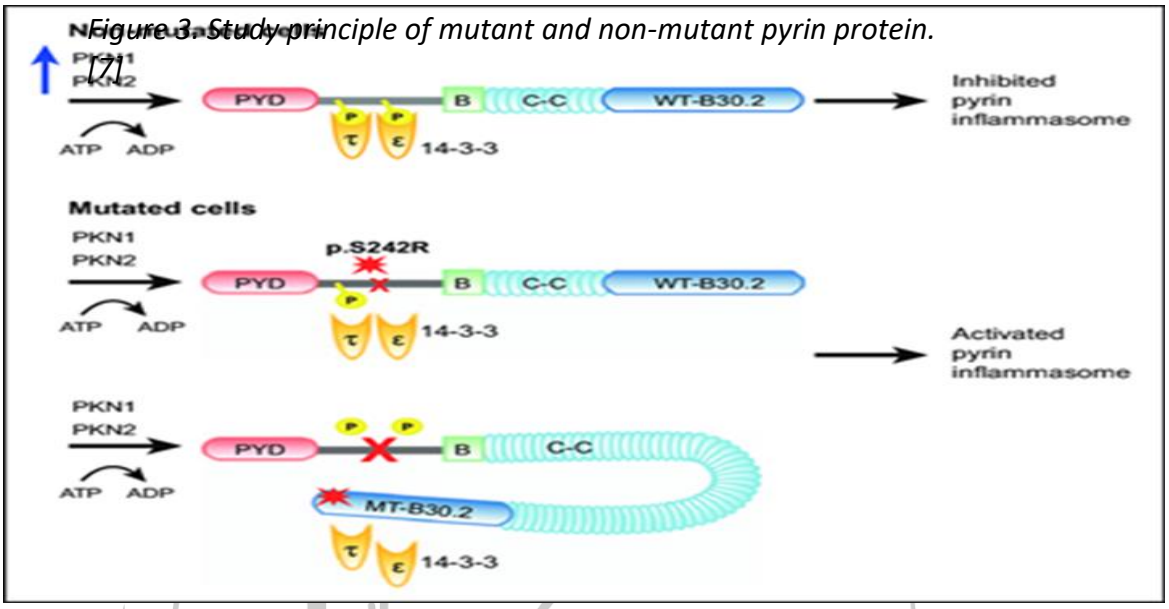


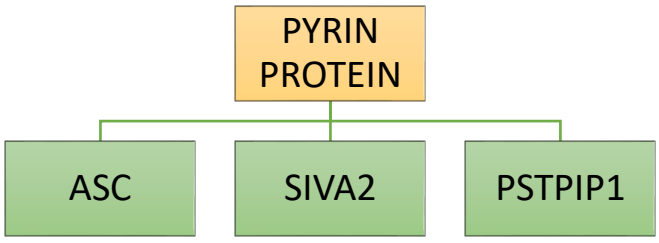
Figure 3. Study principle of mutant and non-mutant pyrin protein. [7]

PKN1 and PKN2 are effector kinases of RHOA that mediate the phosphorylation of pyrin and bind to 14-3-3 inhibitor proteins. Inhibition of pyrin may increase tue agents that activate PKN1/2 or following the release of GEF-H1. If pyrin phosphorylation decreases (due to mutations in the MEFV gene or through bacterial pathogens), the activation of PKN1/2 is also reduced by the defective function of the low-GEF-H1 pathway. This step results in the activation of Pyrin inflammatory tues and the release of mature IL-1β and IL-18. The appropriate stimuli can also lead to a decongestation of inflammation.[8]

They explained that the binding of protein 14-3-3 to rice creates a protection system, and its release from rice activates the protein and increases the production of IL-1 β. 14-3-3, and the protein protection system of rice is due to the phosphorylation of rice by PKN1 and PKN2 at Ser208 and Ser242, which keeps rice inactive.[9]

FMF mutations located on the B30.2 domain at the C-terminal tue of pyrin can also prevent the phosphorylation of pyrin and activate it by blocking the phosphorylation of PKN1 and PKN2, and then facilitate the release of the protective protein 14-3-3 from pyrin.[10]

2.2.2. Proteins Affected by the Mutated Pyrin Protein



When the pyrin-SIVA interaction was examined, it was shown that mefv was 10. the SPRY region encoded from its exon has been shown to be essential for the interaction of a protein region encoded by Siva. The prevalence of MEFV mutations is 10. since it is located in the exon, it is believed that Siva may play a role in the mechanism of occurrence of FMF disease. [11]

It has been shown that the apoptotic effect of Siva decreases with Pyrin. Due to the apoptotic effect of Siva, this interaction was considered to be important for apoptosis. On the other hand, when Pirin and Siva 2 are transferred to a cell together, it has been found that they settle under the cell membrane in the potion, and it is believed that they may play a role in cell migration due to this location.[12]

Another protein associated with pyrin is ASC. ASC has been described as a protein characterized by speck formation. In addition, it has been shown that ASC has a function in inflammatory signaling pathways, is located in multi-molecular structures called inflammasomes, and in addition, ASC is a key protein in the programmed death of macrophages. [13]

In one study, it was shown that Pyrin and ASC are found together in the speck structure. It supports the idea that an increase in the amount of speck occurs when pyrin is mutant, and this is due to the fact that Pyrin and ASC act together in the regulation of inflammation.[13]

It is PSTPIP1 in a protein with which pyrin interacts. PSTPIP1 is one of the regulatory proteins of cell migration. It has been thought that Pyrin protein and PSTPIP1 protein work together to regulate cell migration and therefore play a role in inflammation. Recently, it has been shown that Pyrinin interaction with ASC helps ASC for oligomerization in PSTPIP1.[14]

A mutant Pyrin protein Siva affects the functionality of PSTPIP1 and ASC, leading to various problems in the cell.

2.2.3 The impact of cytokines that creates problems with loss of

Cytokines are peptide or glycoprotein chemical message molecules that mediate hematopoietic cells in the development and regulation of the inflammatory and immune response. Cytokines can be divided into two groups as pro-inflammatory and anti-inflammatory.

Proinflammatory cytokines released at the initial stage of inflammation are necessary for the initiation and maintenance of the immune response.tue.raft. The main pro-inflammatory cytokines involved in inflammatory processes are TNF-α and IL-1.[15]

In october, IL-6 and IL-18 are also secondary auxiliary pro-inflammatory cytokines. IL-1 is mainly responsible for the destructive effects of the disease and carries out proteoglycan and cartilage destruction. One of the subunits of IL-1 is Interleukin-1ß. Caspase-1, which activates Interleukin (IL)-1ß in the presence of mutant Pirin, is unable to perform its function in the inflammation control mechanism. This inflammation of caspase-1, which has been stimulated, cannot be stopped.[16]. TNF-α, on the other hand, is more responsible for proliferative and inflammatory events. There is no significant difference between the TNF dec cell source (LP) and the lipopolysaccharide-activated antigen. TNF-α is secreted by

differentiated T cells with cells involved in the natural immune response, such as macrophages and monocytes. It stimulates the release of TNF-α, IL-1, IL-6, IL-18. It is also a mediator that stimulates the production of IFN-γ and IL-2 by T cells. One of the important cytokines in pathogenesis is IL-6. It plays an important role in increasing acute phase reactants and inflammation in rheumatoid arthritis.

Anti-inflammatory cytokines, on the other hand, are secreted in the advanced stages of inflammation and provide control of the inflammatory response. These are IL-4, IL-10, IL-11 and IL-13. Important roles in the inflammatory response in target cells and cytokines in the activation of regulatory mechanisms that affect the spread of the inflammation and cytokines occurs in a very short time and avoid the unwanted effects in the organism, or the mechanism of inhibition initiates a natural limit.[17]

Mutations in pyrin affect the release of cytokines, so mutations/mutations that occur in the Pyrin protein also cause severe FMF symptoms.

2.3. Clinical Symptoms

As a result of these mechanisms, clinical symptoms of fever, attacks of inflammation, abdominal pain, painful and swollen joints, swollen scrotum, Nov pains and chest and legs, especially the knees under it is characterized by a red rash. These emerging problems negatively affect the patient's daily life.[18]

One of the important complications of FMF patients is amyloidosis, which can lead to kidney failure. Amyloidosis is the most common complication and leads to the accumulation of a protein called Serum Amyloid A in the kidneys. This accumulation is manifested by proteinuria. Recurrent acute attacks in FMF are accompanied by an increase in the protein SAA (Serum Amiolide Amylodiase), which is the acute phase reactant. The fact that the disease lasts for many years, and recurrent acute attacks lead to changes in the structure of this protein, which leads to the storage of amyloid in the kidney and other organs. This accumulation causes nephrotic syndrome, which is observed as excessive protein loss in the urine, and then renal failure. The most common and risky mutation of amyloidosis is M694V.[19] Infertility can be observed when FMF leads to inflammation in the reproductive organs.

The purpose of this project is to correct the mutation in the MEFV gene and to express the correct coding of the Pyrin protein. Therefore, it is necessary to try to solve the problem of individuals diagnosed with FMF.

2.4. The Inadequacy of Existing Solutions For FMF Disease

To treat FMF disease, various drugs are used. The most common of these is colchicine. Although colchicine, having a narrow therapeutic index, is not able to completely prevent febrile attacks, its use can stop the progression of amyloidosis, provoke a positive reaction to inflammatory effects, and in the absence of irreversible glomerular damage, form reverse proteinuria.[20]

In the first month of treatment, up to 10% of patients may experience gastrointestinal discomfort. This condition can lead to increased excretion of starch, fatty and bile acids from the stool, as well as a decrease in the absorption of dxylose and vitamin B12. With the use of this drug, patients show a lack of vitamins. [21]

It has been suggested that colchicine, a molecule that disrupts the microtubule structure, inhibits cell migration through microtubules and provides FMF therapy. As a result of the administration of different concentrations of colchicine in THP-1 cells, it was seen that the expression of MEFV was suppressed(figure 3). The fact that pyrinine is also an actin-related protein has suggested that a decrease in MEFV expression may be due to rearrangement in the actin skeleton. Therefore, it has been thought that colchicine suppresses cell migration by rearranging the actin skeleton. [22]

As can be seen here, the Colchicine drug does not work specifically for pirin portein, which is produced from the MEFV gene, but rather interferes with systems affected by rice or products that will be products of proteins with which rice interacts. Therefore, it is not an exact solution for FMF disease. Over time, resistance to the drug Kolchisisn may develop in some sick individuals.

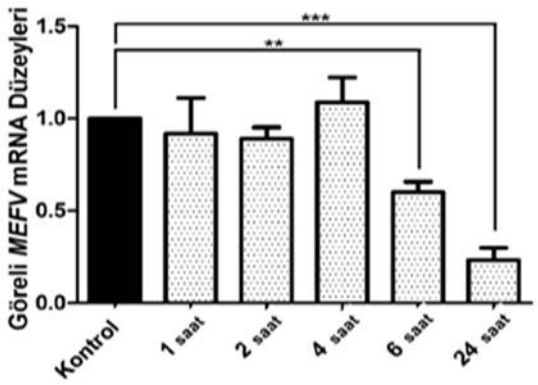


*Figure 3. Farklılaşmış THP-1 hücrelerinde farklı sürelerde kolşisinin uygulanması durumunda MEFV ifadesinin ölçümü. Tüm deney setleri üçlü tekrarlar halinde yapılmıştır.(***p<0.0001, **p<0.001)* [13]

3.Solution

WHY IS CRIPSR A SOLUTION

in the 1970s, with the development of genetic engineering (manipulation of DNA or RNA), a new system of genome editing was formed. Genome editing technologies based on bacterial nucleases (ZFNs, TALENs and CRISPR/cas) have been developed rapidly over the past 10 years. Together with the basic research, successful results have appeared in various fields, ranging from biotechnology and biomedical research. Genome editing provides an opportunity to perform gene "adding, cutting and correcting" in vitro or in vivo.[23][24]

Cas nuclease with clustered regularly spaced short palindromic repeat (CRISPR) is a robust gene editing tool derived from a bacterial adaptive immune defense system. This system can be efficiently programmed to modify the genome of eukaryotic cells (DNA-RNA) and has emerged as a potential alternative to ZFNs and TALENs to induce targeted genetic modifications.[25][26]

Upon recognition of the target DNA sequence, Cas9 mediates the cleavage of the target DNA upstream of PAM to form a double-chain breakage (DSB). This DSB is then repaired by cellular machines via non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways.[27]

NHEJ is the preferred repair pathway in eukaryotic cells; however, this repair mechanism is considered to be imprecise because it simply reconnects two broken ends, which often leads to destructive additions and deletions (indels) at target loci.

The CRISPR-Cas system allows efficient generation of gene knockouts by generating indels in a coding exon. In contrast, the HDR pathway is a more precise mechanism, although it is much less efficient than NHEJ. In particular, HDR provides a repair template containing homology arms surrounding the cleavage site with sister chromatids and artificial DNA genome editing technology that are naturally present during the S and G2 phases of the cell cycle, providing precise insertion of a sequence into the cleavage site.[28]

EXON EXCHANGE BY THE KNOCK-IN METHOD USING A PHYSIOLOGICAL PROMOTER IN THE MEFV GENE

Although pyrin protein is synthesized in various tissues, it is most often synthesized by blood cells (immune system cells). Various methods of gene therapy of the pyrin protein were discussed, and what can be done of these methods for MEFV gene repair was discussed.

First of all, the fact that more than 300 missense mutations are found on the MEFV gene leads to a wide patient scale. Moreover, when researching the disease, we found that FMF mutations are found in exon 2, 3, 5 and exon 10, and the genotype character of different mutations varies in different races, and in some patients there are more than one mutation at the same time.[29]

In general, the mutated Pyrin protein interacts with various molecules and affects the mechanism of pathway functioning. For this reason, gene therapy cannot be done in a regulatory therapy. Given all this, we decided to conduct a knock-in study to provide a treatment solution to patients from all walks of life, rather than a solution that interferes with a single mutation (such as base editing, prime editing), and we aimed to change all the exons of the gene.

We aim to put a functional new MEFV gene by putting the exons of the MEFV gene together with the knock-in method, but since there are no problems with the original promoter part of the MEFV gene, no changes will be made to the promoter part. Immediately after the promoter, the gene will be changed by cutting to target the ki 5'UTR region. Since the promoter will remain constant, there will also be no need for any extra regulation, a healthy energy level will

be maintained. Again, the feasibility of your project will be increased by aiming to get maximum efficiency with minimal intervention.

We will conduct gene therapy Ex vivo. The reason for this to be done ex vivo is that gene therapy of a gene mutated product that is most commonly found in cells expressed in the blood carries risks, the most important of which is toxicity. Conducting a therapy in an individual's blood can make him peak in terms of his immuno profile. But if we work ex vivo with this treatment method, the probability of responding to treatment will increase due to the fact that the person's own blood will be treated, and the mobility that can occur in the immune system will be minimized due to the fact that there will be one-to-one antigens in their own blood.

Using the NCBI and EPD sites, the sequence and promoter sequence of the human MEFV gene were found. After the found seknas, sequences such as gRNA, Cas selection will be selected.

1. GUIDE RNA

gRNA is a complex consisting of two subunits; crispr RNA (crRNA), a 17-20 nucleotide sequence that complements the target DNA, and a tracr RNA that acts as a binding scaffold for the Cas nuclease.[30]

gRNAs can be shortened and fused to result in a 'single guide' RNA (sgRNA).

In order for Cas9 to find and cut the desired region in the MEFV gene, sequences and scores were evaluated and the gRNAs were determined using Benchling software. Of these, off-target and on-target RNAs have been determined to be the most suitable guide RNAs. Three gRNAs have been selected. Selected gRNAs :

gRNA1: GCCTCTCCTGCTCAGCACCA On-Target: 54.4 Off-Target: 31.3

gRNA2: TCTTAGCCATGGTGCTGAGC On-Target: 48.2 Off-Target: 34.3

gRNA3: AGAGGCTCGAGCCAGCTGTC On-Target: 44.9 Off-Target: 37.8

For the most efficient selection of gRNA, the gRNA Cas protein will be transferred to the cell line in the well-platelets. After this application, the most efficient guiding RNAs with western blotting and Flow Cytometry will be selected and used at the ex vivo stage.

CAS9 SELECTION

The Cas9-gRNA complex is aimed at searching for NGG protospacer-adjacent motifs (PAMs) in the genome, and if there is sufficient complementarity between the guide and the target, conformational changes occur, and Cas9, now fully active, decays DNA, making a targeted double-stranded break (DSB). If a homologous donor DNA template is provided during DSB repair, the homologous recombination (HR) repair pathway can produce more precise changes

to the target DNA, including everything from single nucleotide changes to the insertion of large gene cassettes.[31][32]

There are a wide variety of Cas proteins, examples of which are (such as Cas9, Cas13). Cas9 proteins, which have also been modified in various numbers, are mevut.

The most suitable Cas9 protein for the knock-in treatment we are going to do is determined to be HiFi CAS9-R691A. Because HiFi CAS9-R691A will be quite advantageous against the use of other types of Cas9 proteins. The HiFi CAS9-R691A protein stimulates potent gene targeting at therapeutically relevant loci. Although WT (Wild Type) Cas9 proteins increase the knock-in effect, the indel mutation-forming effect is also as high as the off-target effect. Using the HiFi CAS9-R691A, the off-target effect will be achieved up to 20 times lower, while providing an accurate cutting probability equivalent to that of the WT Cas9. In this way, the probability of cutting the correct place of HiFiCas9 accompanied by gRNA will be increased. The number of knock-in cells that will be obtained will also increase. It is expected that the efficiency obtained from the project will be increased.[33]

DONOR DNA

In recent article scans on knock-in, it was seen that using linearized dsDNA donors increases the effectiveness and speed of knock-in. This method is also referred to as TILED-CRISPR (targeted integration with linearized dsDNA-CRISPR) in some articles. It has been shown that a donor DNA transplant with a total volume of up to 6kb can be performed using TILD-CRISPR.[34] This method will not cause any problems in placing a template with the excess volume of the base that it can take with this method, and we will use the advantages of the linerized version.

To the right and left of the donor DNA, homology arms will be placed to increase the level of integration with the gene in the same sequence/sequence as the regions located in the human gene. Literature reviews show that the Knock-in method, whose efficiency is already low, has homology arms added at less than 500 base pairs, which reduces the integration efficiency of the knock-in.[35] Adding 800 base pairs of homology arms in the MEFV gene (46) also corresponds to the UTR region.) It is aimed to increase our knock-in rate.

For the follow-up of the integration of donor DNA, the sequence of the EGF Protein will be inserted before the homolgy steps. Enhanced GFP (EGFP) was produced to increase the fluorescent efficiency of wild-type GFP and the level of expression in mammalian cells. In our project, it will be used to select the cells in which the knock-in is successful by integrating EGFP into the donor DNA.

The P2A sequence will be used to ensure that EGFP is expressed. P2A sequences use a single promoter, but they are used in the production of multiple proteins. Approximately their length dec in the range of 18-22 amino acids. These sequences have the ability to self-destruct. The reason why the P2A array is used in the project is that its efficiency is higher compared to

other 2A arrays.[36] The P2 DEC sequence will be located between the MEFV sequence and the GFP sequence. Because the P2A sequence attaches itself, it will not be expressed in the gene and will not be able to make a false positive result based on sequence results or various tests.

In addition, after adding P2A and EGFP to the Donor DNA template, we omitted the number of stop codons that had been inserted to stop the synthesis of the gene sequence. The goal here is that after the stop code, although the homology arm sequence does not produce a functional protein, homology arms can also be transcribed, even if there is a very low probability that the mutated gene can also be transcribed. It is aimed to minimize this possibility by adding an extra stop code. Because it has a capacity of up to 6kb with TILD-CRISPR, a loop consisting of a total of 4732 base pairs will not be difficult to transfer DNA. The cells where the knock-in is successful will be able to be selected with the help of EGFP.

CELL LINE SELECTION

FMF is known that the disease is most commonly caused by the mutated Pyrin gene produced in the blood. The aim of the project is to produce the blood stem cells that produce the most pyrin protein. In this way, it is aimed to obtain healthy Pyrins, but it cannot live long in the cells produced in the blood, so after a gene therapy is performed there, the effects of the therapy will be over when the cells complete their life. In other words, this should be such a cell line that, in addition to its long life, it should be continuous in expression efficiency. As the cells differentiate, their proliferation capacity decreases and their differentiation (differentiation) increases. That is why what we need to achieve is to knock-in cells that have a constantly self-renewing mechanism. The most suitable cells for these filters are Hematopoietic stem cells.

HCG is a multipotent adult stem cell that can regenerate on its own, has the ability to form cells for a long time, and has the ability to differentiate into more mature cells. hCG cells can form different types of mature cells such as neutrophils, monocytes/macrophages, basophils/mast cells, eosinophils, erythrocytes, platelets, dendritic cells, B and T lymphocytes, NK cells.[37]

Recent studies have shown that genetic errors that cause hematopoietic disease can be corrected in long–term vaccination HSC using CRISPR-CAS9 HSC are also the most appropriate type of cells, since they have the ability to turn into cells containing the MEFV gene.[38]

Stem cells will be isolated from the peripheral blood of sick individuals with FMF. In this way, we aim to show that we can end the symptoms of FMF by directly affecting the immune system.

THE RNP METHOD

The way the Cas protein and the guide RNA were sent should have been a method that would not reduce each other's efficiency, but would have enabled effective and safe access to the nucleus. Although there are methods such as viral, RNA, Transient, the Ribonucleoprotein (RNP) method provides direct transmission. Its most important feature is that it eliminates the

possibility of DNA integration in the host genome compared to the most preferred viral transfer method and strengthens the applications of CRISPR genome modification technology.

RNP transmission, eliminating the need for transcription and translation of intracellular nuclease genome is functional because it allows you to immediately edit the fastest and initially high in cell-genome after editing complexes are rapidly cleared through experience fragmentation, and this editing activity of short-lived makes. The short-term duration of this activity (the presence of RNP) occurring in cells results in highly efficient regulation while minimizing its off-target effects (off-target effects).[37]

3.2. *EX VIVO* STAGES

After the preparatory stages for CRISPR are completed, a knock-in selected Hematopoietic blood cell line will be created that will be performed ex vivo. Peripheral blood is planned to be taken from an individual with FMF for the cell line. In order to be able to select hematopoietic stem cells from the blood taken, CD34+, CD59+, CD90/THY1+, CD38+, C-Kit+ and negative marker kits CD34- will be used to select cells using Flow Cytometry.

After the appropriate cell culture medium is made for the stem cells selected with markers, the continuity of the cell line will be ensured with the primary stem cell culture. The cultured stem cells will be transferred to Hematopoietic stem cells by 4D Nucleofection using the HiFi Cas9-R691A protein + gRNA and ds Loop DNA, which has been made by the RNP method.

3.2.1. 4D Nucleofection

This method is an electroporation method developed for transferring to eukaryotic or mammalian cells. Although it is expensive compared to other methods to transfer using 4D Nucleofection, it will increase the efficiency of Knock-in cells in experiments with low efficiency such as Knock-in.

Because Nucleofector ® Technology uses a special combination of optimized electrical parameters and cell type-specific solutions, it allows the transfer of a molecule directly to the cell nucleus. Nucleofection with superior transfection performance has several advantages over conventional electroporation methods:[39]

1. It maintains the physiological state and vitality of the transfected cells at the highest level.
2. Analysis of the results of transfection allows you to do it shortly after the transfection.
3. It provides transfection of a wide variety of substrates, including DNA, mRNA, miRNA, siRNA, peptides or proteins.
4. It provides for the transfection of difficult-to-transfect cells, including primary cells, stem cells, neurons and cell lines, as well as the transfection of cells in a state of adhesion.

After the transfer, the HiFi-cas9 protein complex recognizes and interrupts the targeted PAM sequence, and the Cyclic DNA is expected to be integrated into the 5'UTR region with HDR(Homology Directed Repair). However, given the off-target effects that exist, although we have used High Fidelity –CAS9, we cannot expect integration to occur correctly in all cells. For this reason, it is aimed to use the Flow Cytometry method only to select the cells in which the Knock-in has been successfully performed. Cell selection will be facilitated because EGFP proteins are added in front of the Homology arms in the downstream of the cyclic DNA. Flow Cytometry using the EGF protein contained in the loop DNA will be used to select the cells. To ensure this, a special Flow Cytometry kit will be used that separates cells with EGFP.

3.2.2. Determination of Dosage

The amount to be applied to the cell line will be determined by finding the IC50 and LD50 values. The appropriate amount of RNP and ds Loop DNA will be applied to the cells.

3.2.3. Tests For the EGFP Protein

In order to prove that Knock-in occurs in selected cells, protein purification will be performed if EGFP is at a sufficient level after measuring the density of the protein in ELISA. It is aimed to support the bands obtained in SDS-PAGE by analyzing the protein bands purified and subjected to SDS-PAGE with a more specific method, Western Blot, to prove that the bands obtained in SDS-PAGE are EGFP. The cells that have been knocked in will be checked.

3.2.4. Tests For Pyrin Protein

The transformation of cyclic DNA into a functional protein is the main purpose of our knock-in. In order to understand whether the Pyrin protein is transcribed in cells, the density of Pyrin will first be checked by ELISA.

After obtaining sufficient density in the ELISA test, the Pyrin protein purification process would be performed. After the purification process, the presence of Pyrin protein will be shown on the SDS-PAGE, and this process will be strengthened with the Western Blot.

3.2.5. Indell Tests For Mutations

Southern Blot, Cleavage Assay and NGS will be performed to determine if indel mutations have occurred after the integration of loop DNA and to prove whether the loop DNA has settled in the right place.

After it is proved that the knock-in occurs in the selected cells, it is necessary to measure the FMF-related cytokine levels in the cells (Neutrophils, Macrophages and Dendritic) that these stem cells are likely to transform into in a living organism.

3.2.6. Tests For The Selection of Cells That Will Transform

It is aimed to separate stem cells from likely cells to be transformed with FACS using specific kits for Neutrophils, Macrophages and Dendritic cells. After this distinction is made, FMF-related cytokines will be looked at again in each free variety. It will be stimulated with bacterial LPS to measure the inflammatory response of cells.

3.2.7. TESTS FOR CYTOKINES

The levels of IL-6 for neutrophils, IL-1B for macrophages and TNF-a cytokines for Dendritic cells will be determined.

In FMF disease, these cytokines are found at a higher level than in healthy people. In order to understand whether the treatment we are doing is correcting this disease, the difference between these levels will be exaggerated.

In order to determine the cells with knock-ins, we will do the tests we do for the Knock-ins that take place in the cells we want.

First, the density of these cytokines will be looked at in ELISA. Protein purification will be performed to show the appropriate density of cytokines on the SDS-PAGE. A Western Blot will be performed to prove that the bands obtained in the SDS-PAGE are the corresponding cytokines. The levels of cytokines will also be measured by qPCR.

3.2.8. Creation of Ex Vivo Experimental Control Groups

Control groups will be determined to make sense of the obtained yields.

Control Group 1: Values of mutant Pyrin cytokines in cells
Control Group 2: Cytokine values after Gene Therapy (Knock-in) in cells
Control Group 3: Pyrin cytokine values in healthy cells

3.2.9. Analysis of the Results

In methods such as Western Blot, Southern Blot, NGS, the weights, densities, amounts of expression of proteins will be compared with the control groups in methods such as Western Blot, Southern Blot, NGS.

Cytokines whose levels are quanitatively determined by qPCR will also be compared. After the tests have been performed, it is expected that the control levels in healthy cells will increase at the same or close rates as the cells that have been knocked-in.

3.3. Experiments in Model Oragnism

A literature review has been conducted for a model organism specific to the MEFV gene that will be used in animal experiments. As a result of literature reviews, it was decided to use a humanized FMF Mouse model. The reason why we chose humanized is that the treatment was planned to be applied in ex-vivo, so we chose it because it was easy/feasible to treat the cells taken from human blood and give them to the model organism. Jakson Laboratory has been contacted for this mouse and the appropriate mouse model has been selected.

Characteristics of the Mouse

The Code of the Mouse you selected is : 129S6/SvEvTac-MEFVTM1BHK/J

In this mouse species, a neo cassette stops gene expression by replacing the entire coding region of the Mediterranean fever (Mefv) gene. Homozygous MEFV-deficient mice are viable and fertile. These mice exhibit increased interleukin (IL)-1β release by macrophages in response to inflammatory stimuli. At the same time, this mouse is also suitable for stimulation with LPS.3.3.1.

In Vivo Experiments

Irradiation will be performed before the cells are transferred to the mouse, and the mouse will be made suitable for transfer. 4 Hours after irradiation, the cells will be injected into the mouse through the tail vein.

Blood will be taken from the mouse to measure cytokine levels in the mouse. The density of cytokines in the received blood will be checked by ELISA. Protein purification will be performed to show cytokines with the appropriate density on the SDS-PAGE.

3.3.2. Gene-level Analyses in Mice

After the ELISA test, the proteins will be purified and subjected to SDS-PAGE. It is aimed to conduct a Western Blot to prove that the bands obtained in the SDS-PAGE are related cytokines. In order to compare the related cytokines with the cytokine levels in the mutated gene and in the healthy gene, it is aimed to perform qPCR to quanitatively determine their levels. After detailed analysis, the data obtained will be compared with the control groups to make sense of the data obtained.

Control Group 1: Cytokine values produced from the mutated gene and activity of proteins with which pyrin interacts

Control Group 2: Cytokine values after Gene Therapy (Knock-in) the activities of proteins with which pyrin interacts

Control Group 3: Healthy Pyrin cytokine values determine the activities of proteins with which pyrin interacts

In methods such as Western Blot, Southern Blot, NGS, the weights of proteins, densities, amounts of expression... will be compared with control groups in methods such as Western Blot, Southern Blot, NGS.

RT-PCR will be performed and quanitatively compared in cytokines whose levels are determined. After the tests have been performed, it is expected that the control levels in healthy cells will increase at the same or close rates as the cells that have been knocked-in.

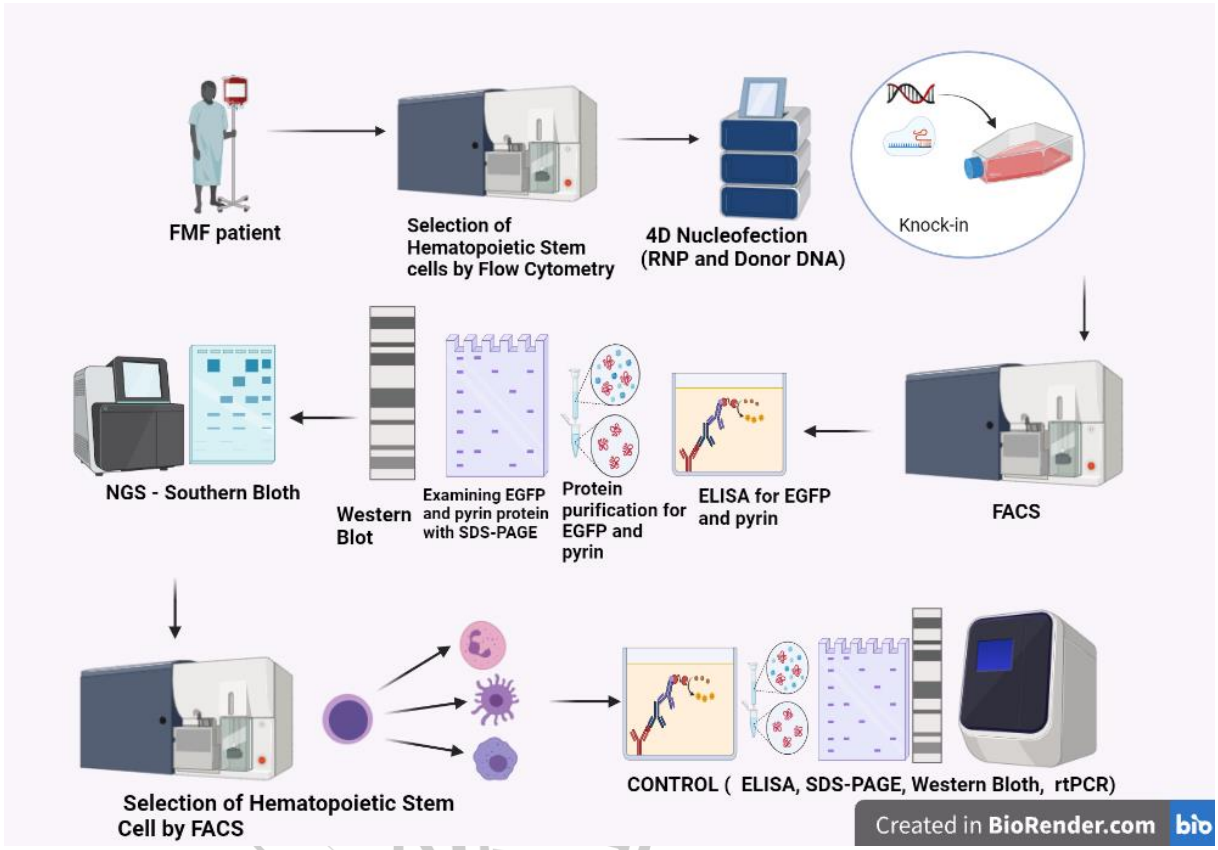
It is expected that cytokine levels will increase in the same or close proportions as levels in healthy humanized mice after the cells Knock-in with CRISPR gene therapy are quanitatively determined with RT-PCR.

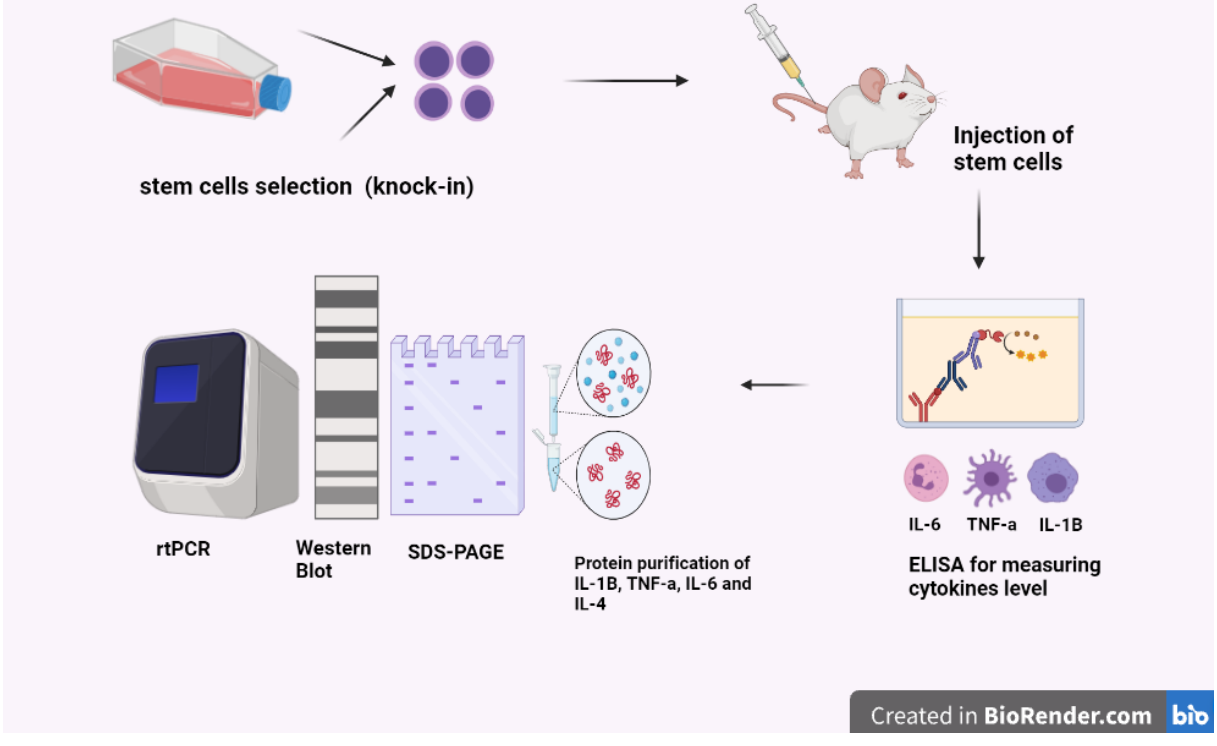
3.3.3. Clinical Symptom Analysis in Mice

Gene therapy with the Knock-in method in mice will be used to check whether FMF symptoms persist to control the effects. After the knock-in application, physical/clinical symptoms will be observed and recorded after gene-level tests and controls. It will be compared with the established control groups.

Mutated Pyrin-bearing creatures also have symptoms such as fever, abdominal pain, visual pain, swelling and rash in the joints. Changes in these symptoms after gene therapy, the effectiveness of terpine will be checked by observing whether there are new symptoms. The effects of terpaini will be analyzed by looking at the C-reactive protein, Fibrinogen, Ceruloplasmin, Haptoglobulin, Serum Amyloid A protein, Erythrocyte sedimentation rate and white sphere values from the blood proteins taken from the model organism.

The normal course will be discussed by comparing these measured values and the observed values with the control groups. It is aimed to continue clinical trials after the analyzes.


FMF patient
Selection of Hematopoietic Stem cells by Flow Cytometry
4D Nucleofection (RNP and Donor DNA)
Knock-in
FACS
ELISA for EGFP and pyrin
Protein purification for EGFP and pyrin
Examining EGFP and pyrin protein with SDS-PAGE
Western Blot
NGS - Southern Bloth
Selection of Hematopoietic Stem Cell by FACS
CONTROL (ELISA, SDS-PAGE, Western Bloth, rtPCR)
Created in BioRender.com


stem cells selection (knock-in)
Injection of stem cells
IL-6
TNF-a
IL-1B
ELISA for measuring cytokines level
Protein purification of IL-1B, TNF-a, IL-6 and IL-4
SDS-PAGE
Western Blot
rtPCR
Created in BioRender.com