Generation and Characterization of Familial Mediterranean Fever (FMF) Disease-Specific Induced Pluripotent Stem Cells (iPSCs)

by

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ABSTRACT

Familial Mediterranean Fever (FMF) is a recessively inherited auto inflammatory disease that is characterized by periodic fevers and local abdominal and joint pain. Mutations in the *MEFV* gene are responsible for the disease phenotype and encodes the Pyrin protein which has a role in regulating the inflammasome and the pro-inflammatory cytokine IL-1β. IL-1β is secreted by various immune cells to recruit other immune cells upon pathogen recognition. While secretion of IL-1β is strictly controlled in healthy people, immune cells of FMF patients secrete high levels of IL-1β. Even though it is demonstrated that various mutations in *MEFV* gene cause abnormal IL-1β secretion, the molecular basis of FMF is still unclear. To elucidate the mechanism of FMF, reliable *in vitro* models of the disease are needed.

Induced pluripotent stem cells (iPSCs) are stem cells that can self-renew while retaining the ability to differentiate. The potential of differentiation, limitless proliferation and the ability to generate them in a patient-specific manner make iPSCs an important source for disease modeling. Disease modeling is the method to generate large numbers of disease-related cell types *in vitro* to analyze the underlying molecular mechanisms. iPSC-based disease models allow for the investigation of disease-associated phenotypes by comparing healthy and patient-derived cells. Such models can also constitute *in vitro* platforms for the discovery of new drugs to treat the diseases in question.

To develop a model of FMF, I generated iPSCs from an FMF patient bearing the homozygous M694V mutation and from the patient's healthy carrier parent as a control. Patient-derived fibroblasts were reprogrammed using episomal vectors to generate integration-free iPSCs. FMF-iPSCs were characterized by gene expression analyses, immunofluorescence staining and teratoma formation assay, all of which indicated that reprogrammed cells are bona fide iPSCs. To generate macrophages which are one of the FMF-relevant cell type from these iPSCs, three different methods including embryoid body formation, co-culture with OP9 cells and viral transfection have been tested but were unsuccessful. Further experiments are needed to generate *in vitro* derived functional macrophage from iPSCs.

FMF-specific iPSCs can be used in the future studies to model this disease. Both healthy and patient iPSCs can be differentiated into FMF-related cell types including macrophages and neutrophils to discover the underlying mechanism of disease. In addition, these cells lines can be utilized in combination with drug screening and genome targeting strategies to open up new approaches to treat FMF disease.

ÖZET

Ailesel Akdeniz Atesi (FMF) otosomal resesif bir oto-inflamatuar hastalıktır. FMF, ataklar halinde görülen ateş, karın ve eklem ağrılarıyla karakterize bir immun sistem hastalığıdır. Makrofajlar bir patojen ile karşılarştıklarında, bir proinflammatuar sitokin olan IL-1β salgılayarak diğer immun hücrelerini aktive eder ve inflamasyonu başlatırlar. Sağlıklı insanların makrofajlarında IL-1β salgılanması sıkı bir şekilde kontrol edilir. Fakat FMF hastalarının makrofajları, *MEFV* genindeki mutasyonlardandan dolayı IL-1β salgılanmasını kontrol edemez ve sağlıklı makrofajlara göre daha fazla miktarda IL-1β salgılarlar.

Uyarılmış pluripotent kök hücreler (uPKH), çoğalabilen ve üç germ tabakasındaki hücrelere farklılaşabilen kök hücrelerdir. Sahip oldukları özellikler uPKH'leri hastalık modellemesi için önemli bir kaynak haline getirmiştir. Hastalık modellemesi, genetik hastalıkların moleküler mekanizmasını anlamak, yeni ilaçlar keşfetmek ve terapide kullanabilmek için hastalıkla ilgili hücre türlerinin in vitro olarak çoğaltılmasıdır. Bir çok somatik hücrenin in vitro olarak çoğaltılması mümkün olmadığı için, hastalardan alınan somatik hücreler önce uPKH'lere programlanıp daha sonra hastalıkla ilgili istenen hücre tipine farklılaştırılır. Böylece sağlıklı ve hastalıkla ilgili mutasyonu taşıyan farklılaşmış hücreler analiz edilerek hastalıklara neden olan mekanizmalar anlaşılabilecektir.

Bu tez çalışmasında, FMF hastalığını modellemek için, MEFV geninde M694V mutasyonu taşıyan FMF hastasından, hastanın sağlıklı babasından ve normal sağlıklı bir bireyden uPKH hattları üretildi. Gen integresyonu olmayan uPKH üretebilmek için somatik hücreler episomal vektörler ile yeniden programlandı. Yeniden programlanan hücrelerin uPKH olduklarını göstermek için gen ekspresyon analizi, immunofloresan boyama ve teratoma oluşturma gibi farklı karekterizasyon testleri yapıldı. Bu testler yeniden programlanan hücrelerin gerçek iPKH olduklarını gösterdi. Üretilen uPKH'leri FMF hastalığından etkilenen hücrelerden biri olan makrofaja dönüştürmek için embryoid body oluşumu, OP9 kokültürü ve virus infeksiyonu gibi farklı methodlar kullanıldı. Fakat hiçbir protokolde uygun miktarlarda makrofaj oluşumu gözlemlenmedi.

Sonuç olarak bu çalışmada, üretilen FMF uPKH'ler gelecekte hastalık modellemesi için kullanılabilir. Sağlıklı ve hasta uPKH'ler makrofajlara veya FMF ile ilgili diğer hücrelere, örneğin nötrofillere farklılaştırılarak FMF hastalığına neden olan mekanizmaların keşfedilmesinde kullanılabilirler. Ayrıca üretilen hücre hattları, gelecekte ilaç taramaları ve Crispr/Cas9 gibi yeni genetik mühendisliği araçları ile tedavi yöntemleri geliştirmek için de kullanılabilir.

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CHAPTER 1

Review of Literature

1.1 Introduction To Familial Mediterranean Fever (FMF) Disease

Familial Mediterranean Fever (FMF) is a recessively inherited auto inflammatory disease. The disease is characterized with periodic fever episodes, abdominal and joint pain, and cause to an acute phase response. As its name indicates, FMF primarily affects people in Eastern Mediterranean countries [1]. FMF shows an ethnic distribution. The prevalence of the disease is high among the Turks, Armenians, Jews, Italians and Arabs [1]. However, the disease is the mostly seen in Turkey [2]. According to studies, FMF is observed more frequently in Greece, Cyprus and Italy than previously believed [3]. Interestingly, the disease has also been identified in Brazil [4]. The gene, responsible for the disease is the MEFV gene (Mediterranean Fever) and encodes a protein called Pyrin [5], [6]. Even though mutations in the MEFV gene are known, the mechanism of the disease is not yet clear. However, it is mostly thought that Pyrin is an anti-inflammatory protein. While wild type Pyrin inactivates caspase-1, mutated Pyrin does not inactivate which in turn causes more activated caspase-1 (formerly called IL-1 beta converting enzyme (ICE)) [5], [7], [8]. Several cells are affected from FMF disease including macrophages and neutrophils. Affected immune cells in FMF patients by MEFV mutation secretes more cytokines including IL-6 and IL-1β than healthy macrophages and neutrophils [6]–[9]. Higher level of IL-1β during inflammation attacks in the body is one of the phenotype of FMF [12]. Even though FMF is an autosomal disease, a slight male dominance is observed [13].

1.1.1 Molecular Mechanism

1.1.1.1 *MEFV* Gene

The *MEFV* gene consists of 10 exons on the short arm of chromosome 16. Several mutations have been identified in exon 10 (for instance, M680I, M694V, M694I and V726A). Mutations have also been identified in other exons such as exon 2. A detailed list of mutations in exons are shown in **Figure 1**. Mutations in *MEFV* gene are mainly point mutations (missense mutations) that lead to single-nucleotide changes. Currently, more than 250 mutations have been identified. Approximately 80% of FMF patients carry one of the M680I, M694V, M694I and V726A mutations [8]. However, some of the mutations are rare

and do not cause any clinical phenotype. The rare existence of such mutations, for instance E148Q, and their contribution to FMF phenotype have led some researchers to claim that these mutations should not be considered as mutation, but rather a polymorphism [14]. M694V mutations present with the most severe form of the disease that can causes serious complications and AA amyloidosis which is accumulation of S amyloid A protein which may cause the kidney failure. All of these identified mutations and complex phenotypes make FMF disease a more complex disease than previously believed.

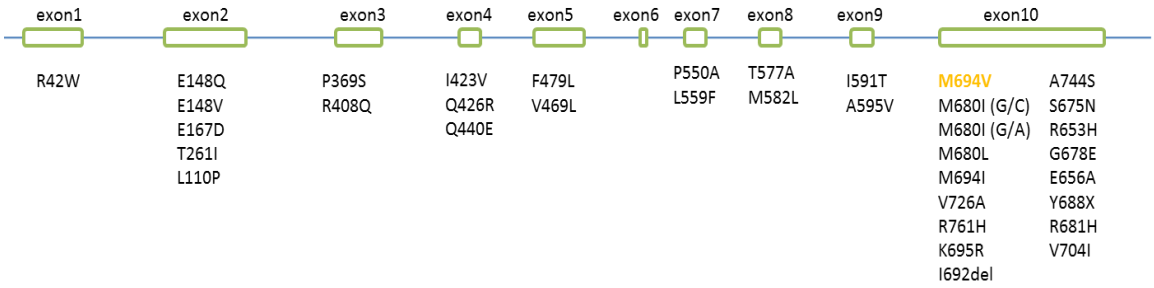


Figure 1: Several mutations have been identified on different exons of *MEFV* gene. Adapted from reference [2]. “Infevers” which is a Human Genome Variation Society (HGVS) affiliated database.(Database website address : http://fmf.igh.cnrs.fr/ISSAID/infevers/)

1.1.1.2 Pyrin Protein

The *MEFV* gene encodes a protein which is named Pyrin by the International FMF Consortium and Marenostrin by the French FMF Consortium [15], [16]. Pyrin has a molecular weight of 95 kDa and is expressed in various cell types including neutrophils, fibroblasts, dendritic cells, eosinophils and monocytes [2], [17]. It is suggested that Pyrin has various roles in the regulation of apoptosis, inflammation and cytokines. Currently, the physiological role of Pyrin is still uncertain in FMF disease. However, it is believed that the primary function of Pyrin is to suppress the inflammatory response [5], [18], [19].

The protein consists of 781 amino acids and contains a Pyrin domain (PYD) which is located at the N-terminal end; the B box zinc finger, alpha helix and a B30.2 that is located at the carboxyl end **(Figure 2).** Even though two nuclear localization motifs exist in Pyrin, this protein does not have any DNA-binding activity. A specific N-terminal fragment of Pyrin translocates to the nucleus after cleavage by caspase-1. This cleaved N-terminal of Pyrin can activate NF-κB [5], [18].

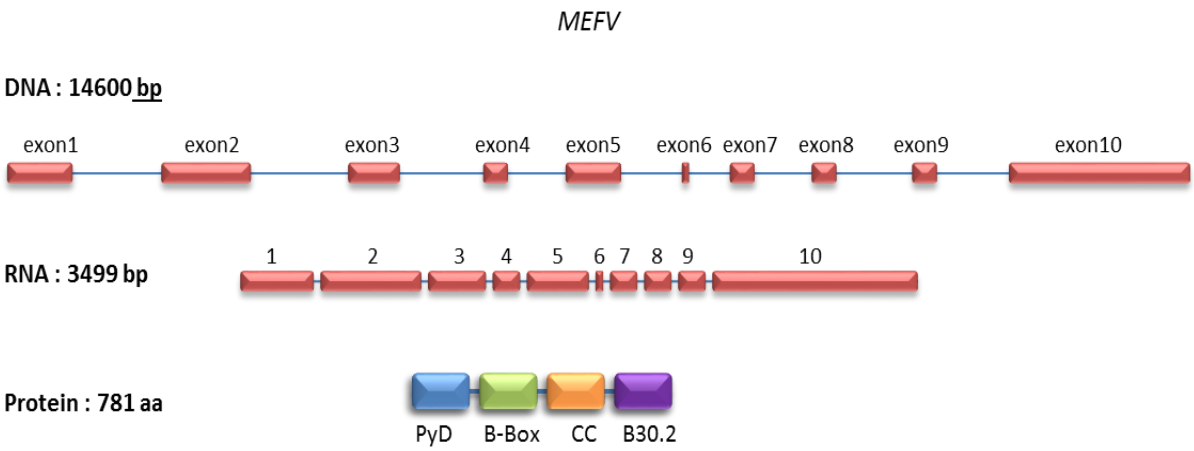


Figure 2: Illustration of DNA, RNA and protein structure of *MEFV* gene. *MEFV* consists of 10 exons that are postranscriptionally modified. Translation of *MEFV* RNA give rise Pyrin protein that has various domains including Pyrin, B-Box Zinc Finger, Coiled Coil and B30.2. Adapted from reference [8].

In contrast to monocytes; in granulocytes and dendritic cells, endogenous Pyrin localizes into the nucleus [18]. Full length Pyrin is cytoplasmic; however, an alternatively spliced form lacking exon 2 localizes into nucleus. In monocytes, Pyrin which is in the cytoplasm, interacts co-localizes with tubulin monomers. This interaction may explain the effect of colchicine treatment in FMF patients [5],[21],[20].

Several domains including a B-box zinc finger (that could be play a role in protein-protein interaction), an α-helical region (which may form a coiled coil configuration) and a C-terminal B30.2 domain (that has a function at ligand binding or signal transduction) can be found in the C-terminal half of the Pyrin between 300-781 amino acids. Also these elements define a family which is TRIM that has an important role in subcellular localization of the Pyrin [21] **(Figure 3).**

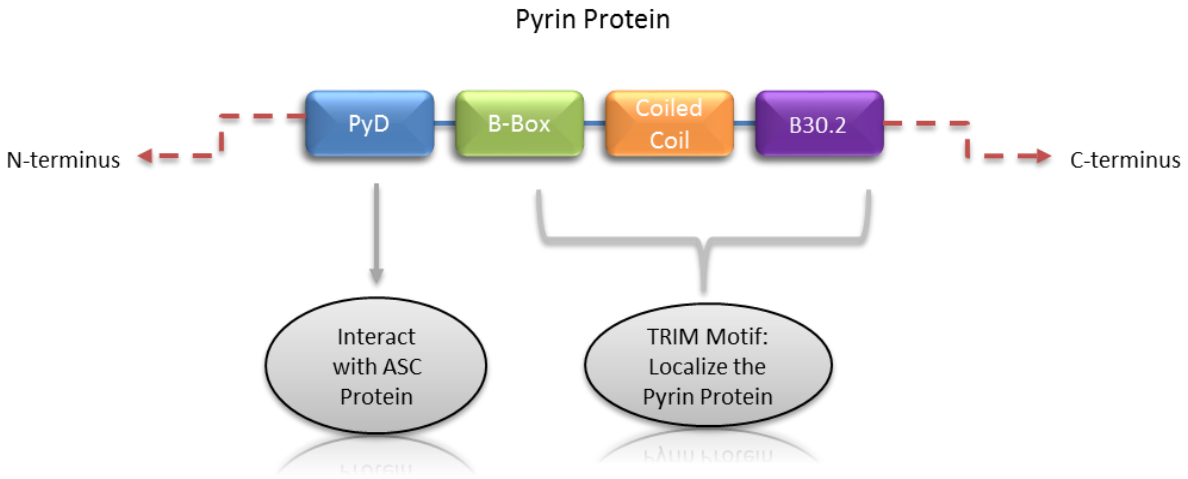


Figure 3: Structure of Pyrin protein. Pyrin domain plays a role in ASC interaction. TRIM motif which consists of B-Box, Coiled Coil and B30.2 may lead the localization of pyrin protein. Adapted from reference [2]

On the other hand, the N-terminal of Pyrin was thought to be a unique domain. However, new studies have revealed that several proteins possess similar sequences with the N-terminal of Pyrin. Hence, the domain of N-terminal is called the Pyrin domain (PyD) [22]. Proteins that contain the Pyrin domain are ASC (apoptosis speck protein with a caspase recruitment domain (CARD)), zebrafish caspase 13, nucleotide-binding site protein 1 (NBS1) and a number of protein that are interferon-inducible, including AIM2 and MNDA. Furthermore, proteins which contain Pyrin domain are thought to be functional in apoptosis, inflammation and innate immunity [23].

1.1.1.3 Interaction Of Pyrin With Inflammasome Proteins

Inflammation is the reaction of the host's immune system to eliminate pathogens. It is characterized with a massive influx of immune cells into an infected or damaged region. Migration of immune cells is under the control of various cells, inflammatory and anti-inflammatory cytokines. One of the pro-inflammatory cytokine that recruits immune cells is IL-1β which is secreted from various immune cells including neutrophils and macrophages [14], [24], [25]. Release of IL-1β is regulated at various steps including transcription, posttranslational processing and receptor binding. In the case of FMF, IL-1β is secreted at higher levels in patients than healthy people during attacks [7], [26]. IL-1β, with a weight of

34 kDa, is synthesized as pro-IL-1β [5]. The immature form of IL-1β (pro-IL-1β) is transcribed upon a primary pro-inflammatory stimuli such as bacterial lipopolysaccharide [5]. Activation of TLR4 by LPS, extracellular matrix or heat shock proteins also cause NF-κB expression which eventually leads pro-IL-1β synthesis [27]. Pro-IL-1β found in the cytoplasm does not have any functional role in the regulation of inflammation. In order to be activated, Pro-IL-1β needs to be cleaved by active caspase-1 to be secreted as a mature form with a weight of 18 kDa.

Activation of caspase-1 is under the control of protein complexes which are known as inflammasomes. Inflammasomes are multi-protein complexes that recognize damage-associated molecular patterns (DAMP) by members of Nod-like receptor (NLR), the family of cytosolic pattern recognition receptors (PRR) [24]. Several inflammasomes with various subunits exist and cleave different caspases to activate caspases. As a result, inflammasomes may have distinct roles in different pathways. The inflammasome that cleaves and activates caspase-1 consist of NLRs, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)) and caspase-1 [28], [29], [30]. The inflammasome becomes activated with the recognition of intracellular danger associated molecular patterns (DAMP) by a cytosolic NLR such as NLRP3 **(Figure 4)**. NLRs then undergo P2X7-dependent (via ATP) or P2X7-independent oligomerization which results in recruitment of ASC by PYD-PYD interaction. ASC also recruits caspase-1 through the CARD domain. NLR, ASC and caspase-1 multi-protein complex keep other caspase-1 proteins in a close proximity so that caspase-1 molecules begins to cleave each other. Therefore, at the end of inflammasome activation, caspase-1 is cleaved; in other words, caspase-1 becomes activated and cleaves pro-IL-1β to form mature IL-1β. Mature IL-1β is secreted from cells and it recruits immune cells to start inflammation [31]. Hence, dysregulation of inflammasome leads to higher caspase-1 activation and causes increased IL-1β secretion which as a result, causes the FMF disease [32].

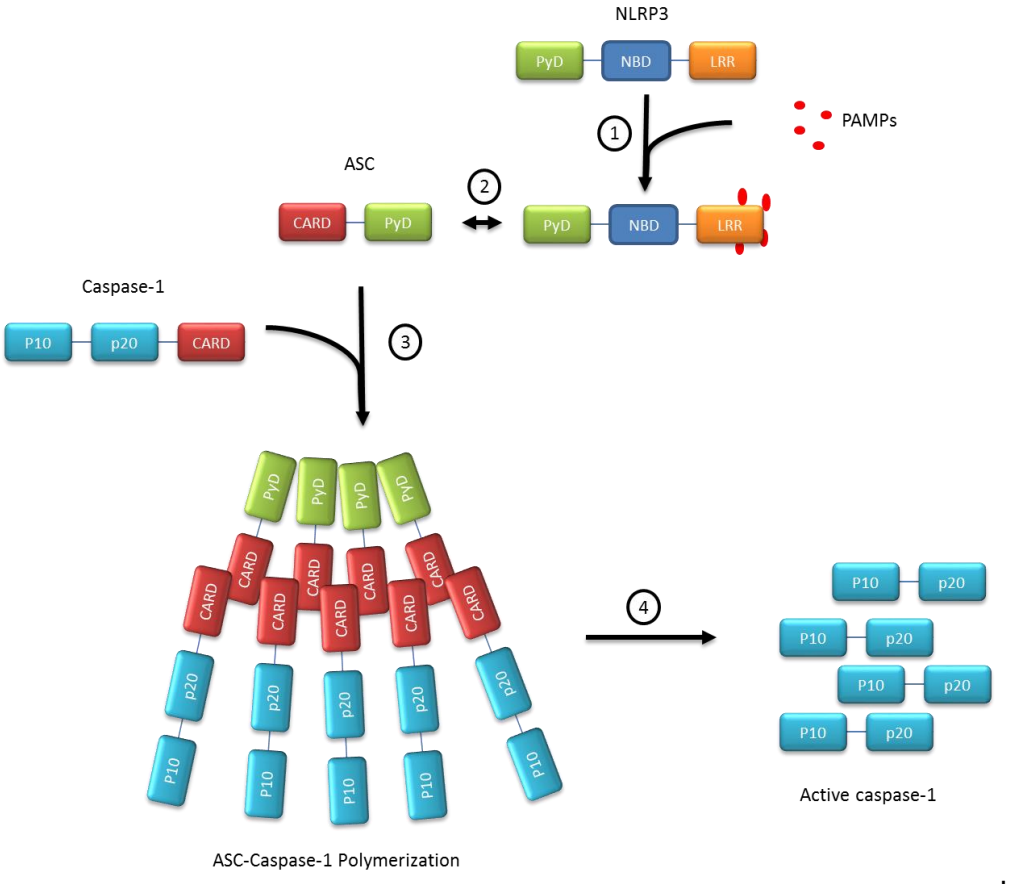


Figure 4: Caspase-1 activation via ASC-NLRP3 interaction. **1)** Interaction of NLRP3, ASC and Caspase-1. PAMPs, such as LPS, stimulate NLRP3 through LLR domain. **2)** Activated NLRP3 recruits ASC using PyD-PyD interaction. **3)** Recruited ASC form a complex with pro-caspase-1 with CARD domain interaction. **4)** Pro-caspase-1 and ASC complex lead cleavage of pro-caspase-1 into active caspase-1. Adapted from reference [30]

Regulation of inflammasome and caspase-1 is carried out by various molecules including Pyrin. Although the exact role of Pyrin in this process is not clear, most studies suggest that wild type Pyrin suppresses caspase-1 activation, however in the mutant form this activity is diminished. It is suggested stimulated immune cells including macrophages and neutrophils in FMF patients, mutated Pyrin cannot suppress active caspase-1 which leads the secretion of higher levels of IL-1β [5], [7], [18], [32].

However, there is still controversy about the role of the Pyrin. The studies have showed different effect of Pyrin on caspase-1 regulation. According to majority of studies, wild type Pyrin suppresses caspase-1 [5] [19]. In contrast, some studies have showed that Pyrin binds to ASC and activates caspase-1 [33]. Chae and colleagues showed that pyrin represses the active caspase-1 **(Figure 5)** [5]. They immunoprecipitated pyrin from THP-1 cells (monocytic cell line) and demonstrated an interaction between pyrin and caspase-1. However, in monocytic cells, there are other proteins that mediate the caspase-1-pyrin interaction such as ASC. To evaluate a direct effect of pyrin on caspase-1 activation, they transfected NIH 3T3-derived PT67 cells, which do not express ASC, with a bicistronic caspase-1/IL-1β construct and a pyrin-expression vector. They generated a cell line which express the same amount of caspase-1/IL-1β construct. However, cells were then transfected with increased amount of wild type and mutant pyrin expressing vectors. Results demonstrated that both mutant and wild type pyrin expressing vector-infected cells secreted less IL-1β. However, wild type pyrin expressing cells had significantly reduced IL-1β secretion than the mutant form. Therefore, the study showed that wild type pyrin suppresses active caspase-1 and decreases IL-1β cleavage.

In contrast to findings of Chae et al., Yu and colleagues indicate that pyrin is a pro-inflammatory protein that support caspase-1 activation **(Figure 6)** [33]. This study showed that pyrin has an important role in recruiting ASC and pro-caspase-1 to initiate self-cleavage of caspase-1. They generated 293T cells that express ASC and pro-caspase-1. Infection of ASC-Caspase-1 expressing cells with increased amount of wild type and mutant pyrin yielded active caspase-1. Thus, they concluded that pyrin does not inhibit caspase-1, and in fact, induces ASC-dependent caspase-1 activation. In this study, mutant pyrin showed similar effects compared to wild type pyrin on caspase-1 activation. Hence, these researchers claimed that pyrin could have a role in the pathogen associated molecular pattern recognition. According to this study, mutant pyrin acquire a gain of function mutation which make mutant pyrin more sensitive to pathogens than wild type. More sensitive pyrin can induce more ASC-dependent caspase-1 activation than wild type pyrin which causes FMF disease.

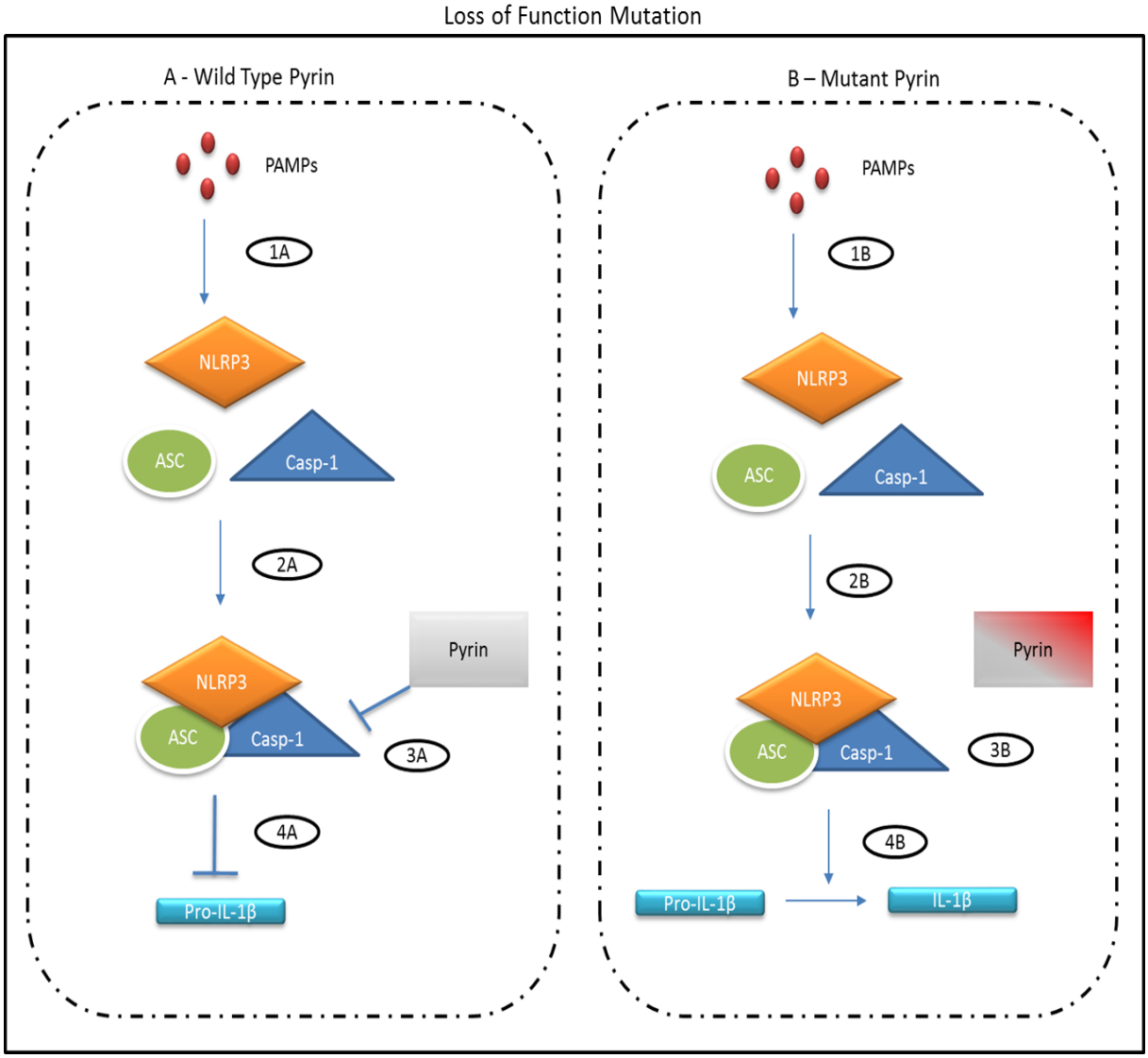


Figure 5: Proposed function of wild type and mutant pyrin as anti-inflammatory protein. **1A and 1B:** PAMPs are recognized by NLRP3 which activates NLRP3. **2A and 2B :** Activated NLRP3 recruits ASC and Caspase-1 that forms NLRP-3 inflammasome. NLRP3 inflammasome then cleaves pro-IL-1β into mature IL-1β. **3A:** In healthy individuals, NLRP3 inflammasome is controlled via Pyrin protein. **4A:** Pyrin inhibits the inflammasome to supress IL-1β cleavage. **3B:** In contrast, mutated pyrin cannot suppress the inflammasome. **4B**: Since the inflammasome is not suppressed by the pyrin, IL-1β is secreted in an uncontrolled manner.

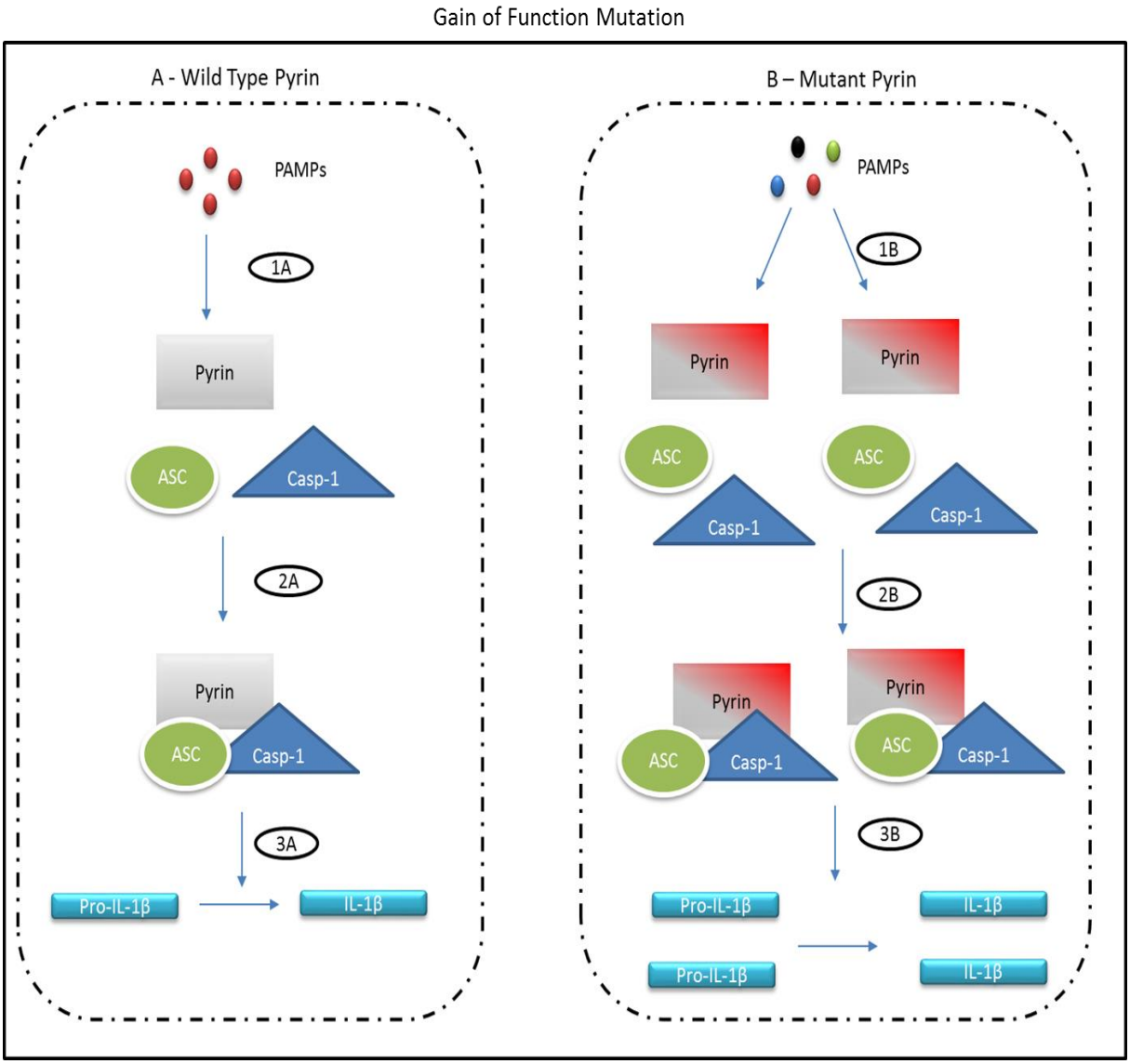


Figure 6: Proposed function of wild type and mutant pyrin as pro-inflammatory protein. **1A:** Pyrin protein may recognize PAMPs through B30.2 domain. However, mutations in Pyrin protein, specifically in B30.2 domain, make B30.2 domain more sensitive to wide variety of PAMPs. Therefore, mutant pyrin protein could be easily activated. **2A** Activation of pyrin leads formation of Pyrin-ASC-Caspase-1 complex. **3A:** Formation of the complex cleaves the caspase-1 into active form of caspase-1. Active caspase-1 cleaves pro-IL-1β into secreted form of IL-1β which initiates the inflammation. **2B and 3B:** However, mutant form of pyrin can be easily activated by PAMPs . Hence, activated mutant macrophages secrete more IL-1β.

Another reason of complexity of FMF disease is the presence of alternatively spliced forms of the Pyrin. As explained previously, several studies have investigated role of Pyrin in inflammation from different perspectives. However, the described functions may be a result of spliced forms of Pyrin. Since spliced forms in these studies may be different, each result may reflect the function of one isoform of Pyrin. Currently more than 10 isoforms are identified **(Figure 7)** [8] [34].

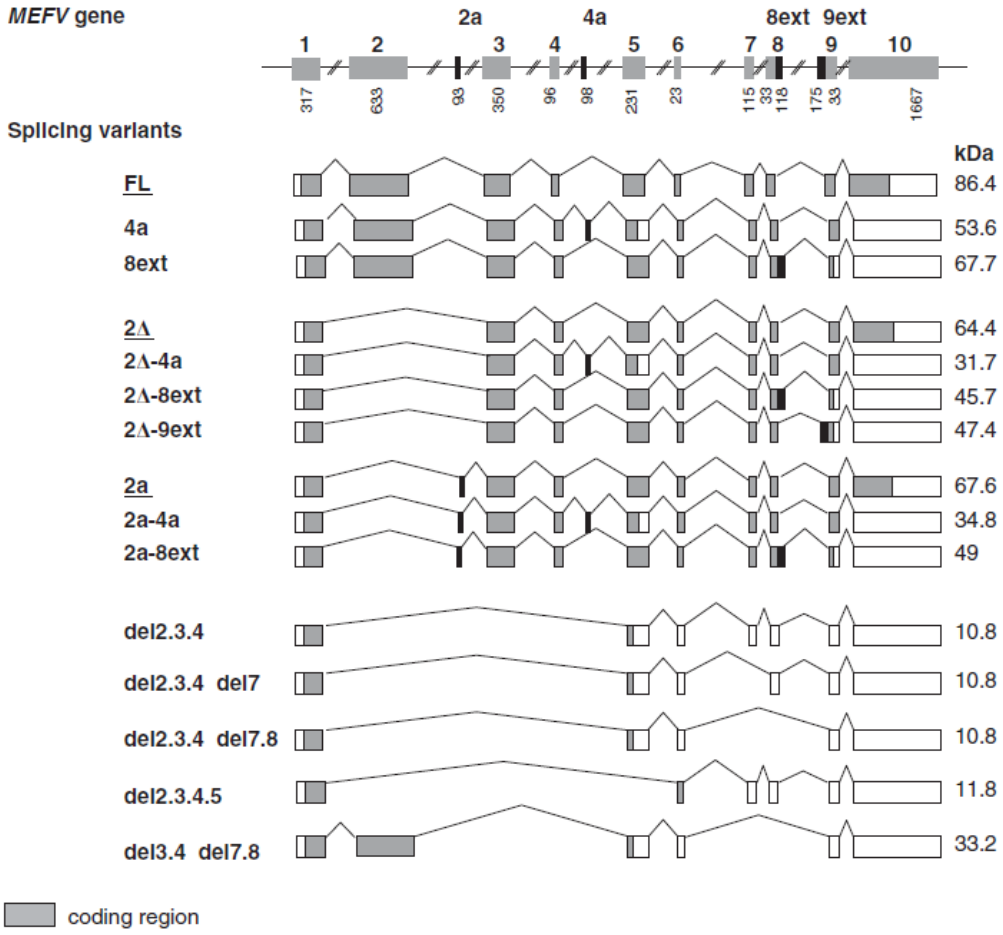


Figure 7: Alternative splicing forms of MEFV gene. [Copyrights of figure could be found in Appendix 1, [8]]

IL-1β gene is located on chromosome 2 and transcribed as pro-IL-1β, with a weight of 31 kDa, which is not physiologically active. Pro-IL-1β has to be cleaved by active caspase-1 to be functional. Active IL-1β is a pro-inflammatory cytokine with a weight of 18 kDa. The cytokine is transcribed in a cell specific manner, mainly from monocytes, macrophages B, lymphocytes, neutrophils, natural killer and dendritic cells. It is demonstrated that

macrophages which are one of the cell type that are affected in FMF patients, release significantly higher level of IL-1β than healthy people during inflammation [25].

In order to synthesize pro-IL-1β, Toll-like receptors (TLRs) have to be stimulated via microbial products such as LPS. However, TLR stimulation is necessary for macrophages but not needed for monocytes, since monocytes can synthesize immature form of IL-1β continuously [35]. Following LPS stimulation, pro-IL-1β is accumulated in cytosol. In the presence of active caspase-1, pro-IL-1β is cleaved to its secreted form, mature IL-1β.

Overall, secretion of IL-1β from macrophages requires two independent signals, first one is pathogen associated molecular pattern recognition via TLR (mostly by TLR4) for pro-IL-1β synthesis, and second one is P2X7 or NLR (especially NLRP3) activation for inflammasome, hence caspase-1 activation [31].

1.1.2 Treatment Of FMF

FMF patients are treated with colchicine as soon as diagnosis is confirmed since colchicine can prevent both inflammatory attacks and amyloid deposition. Generally, colchicine is used orally 1-2 mg/day in adults and 0.5-1 mg/day in children according to age and weight [36].

However, colchicine does not cure FMF, but it prevents the complications of disease and is therefore generally used throughout life. It is also worth noting that colchicine is not effective when it is only used during attacks. Longer time is needed for colchicine treatment to reduce FMF attacks.

Colchicine is a mitotic inhibitor that works by inhibiting beta-tubulin polymerization into microtubules. Besides its mitotic inhibitory effect, colchicine also inhibits inflammasome activation which regulates the IL-1β secretion in monocytes, macrophages and neutrophils. Due to the microtubule disruption, microtubule-mediated transport of inflammasome proteins is impaired in colchicine treated cells [37]. Therefore, co-localization of NLRP3 and ASC is disrupted and cannot lead inlammasome formation which explains the inhibitor effect of colchicine treatment on inflammasomes. It is suggested that stabilization of microtubules in the colchicine treated cells, Pyrin sticks on the tubulin monomers, thus cannot activate caspase-1 [38]. In addition colchicine prevents activation, degranulation and migration of neutrophils which makes colchicine an anti-inflammatory drug [38].

However, some patients do not respond to colchicine treatment. Individuals who are resistant are focus of new therapies such as anakinra, an IL-1-receptor inhibitor and canakinumab, an anti-IL-1β monoclonal antibody [39], [40]. In contrast to Colchicine, canakinumab targets the secreted form of IL-1β. In the case of high levels of IL-1β, canakinumab binds to IL-1β. Therefore, the levels of biologically active IL-1β in patients is controlled [41]. On the other hand, anakinra targets IL-1β receptor to block receptor activation. Even though the abundant IL-1β is secreted from macrophages, it cannot activate other immune cells which have blocked IL-1β receptor due to anakinra [42].

1.2 Introduction To Induced Pluripotent Stem Cells (iPSCs)

1.2.1 Stem Cells

Stem cells are self-renewing cells that can differentiate into terminally differentiated cell types. Stem cells can be isolated from various tissues. According to differentiation abilities, stem cells have different potencies such as totipotent, pluripotent, multipotent and unipotent. Cells that are isolated from 4 or 8-cell stage embryo are totipotent; they can differentiate into three germ layers and can give rise to the entire embryo. Embryonic stem cells (ESCs), isolated from blastocysts, are pluripotent stem cells which can differentiate into three germ layers (endoderm, mesoderm and ectoderm) but cannot generate extra-embryonic lineages. Because of the pluripotency ability, ESCs are capable of generating all three germ layer cells *in vitro* under proper conditions [43]–[45]. On the other hand, adult stem cells such as mesenchymal stem cells (MSCs) can differentiate into a limited number of cell types and are therefore called multipotent stem cells.

1.2.2 Induced Pluripotent Stem Cells And Reprogramming

Induced pluripotent stem cells (iPSCs) have been generated from mouse somatic cells by Takahashi and Yamanaka in 2006 and from human somatic cells in 2007 [46] [47]. To generate such cells pluripotency-related genes were introduced into mouse fibroblasts. Introduction of 24 pluripotency-related genes yielded colonies, similar to embryonic stem cell (ESC) cells. Genes were then gradually eliminated to identify those minimally required to generate ESCs-like cells. Four genes which include Oct4, Sox2, c-Myc and Klf4 were found to be required transcription factors to reprogram fibroblasts into ESCs-like cells. It was demonstrated that these colonies could proliferate for extended periods of time in cell culture

and were able to differentiate into cell types of three major germ layers. These ESC-like cells were called induced pluripotent stem cells (iPSCs) [46], [47].

1.2.3 iPSCs Derivation Methods

In 2006, Yamanaka used retroviral system to introduce four transcription factor into fibroblasts for reprogramming. In recent years, other iPSCs derivation systems including lentivirus, Sendai virus, episomal vectors and proteins have been developed [48]–[54]. Each system has its own advantages and disadvantages. Due to active methyltransferases in iPSCs, viral transcription is suppressed in reprogrammed cells. This suppression is partial, meaning that still some viral, exogenous, transcription factors are expressed. In order to avoid exogenous pluripotent gene expression in reprogrammed cells, other vector systems have been developed such as inducible lentiviruses [55]. Retro- and lentiviruses integrate into host genome and random integration of viral genome can lead to mutations or oncogene activations. Therefore, integration systems are less valuable for clinical purposes. To eliminate integration related mutations in the host, integration-free systems have been developed [56].

Adenoviruses have been used in reprogramming to eliminate integration. However, usage of adenovirus for reprogramming is relatively inefficient compared to integration-based methods. It is also difficult to generate virus-free iPSCs lines using non-integration virus methods [53]. Protein introduction into somatic cells is another method to reprogram cells without genomic integration. However, reprogramming efficiency is very low and it is not a cost-effective method to generate iPSCs [49], [50].

Beyond the viral systems, other methods have also been developed including introducing episomal vectors, proteins and mRNA [57]. Episomal vector is one of the convenient systems to generate iPSCs for therapeutic purposes. Episomal vectors are plasmid vectors that are sustained in the cytoplasm, they do not integrate into host genome. After 10-20 passage, episomal vectors are eliminated from iPSCs [52].

In 2006, when Yamanaka generated reprogrammed cells, iPSCs were cultured on mitotically inactivated mouse embryonic fibroblasts (mito-MEFs). In order to keep iPSCs as undifferentiated cells, an undefined medium which includes animal serum was also used. However, undefined medium and mito-MEFs usage may lead to inconsistent results in clinical applications. Therefore, defined mediums have been produced. In addition, new

matrix compounds were utilized to generate feeder-free iPSCs [58] [59]. Currently, several defined medium and matrix compounds are used to culture iPSCs. Thus, iPSCs that are generated and cultured in animal-free products have the potential to be used in therapeutic approaches.

1.2.4 iPSCs In Disease Modeling

Reprogramming of somatic cells to pluripotent stem cells promises fascinating opportunities to understand mechanisms of genetic diseases. Derivation of somatic cells from a patient with genetic disease leads patient iPSCs which have the same genetic background with patient. iPSCs differentiation into disease-related cell types could be a remarkable method to analyze mechanisms of the cells with disease, in vitro. The process which patient somatic cell reprogramming into iPSCs and differentiation into disease-related cell type are called disease modeling. Differentiated cell types could elucidate molecular mechanism of diseases or could be tested in drug screening to reveal possible drugs (Figure 8). [60]

Disease	Defect	Correction	References
β-Thalassaemia	Deletion in βglobin gene	Gene correction reversed the phenotype	[70]
Bowl disease	Unknown	-	[62]
CINCA syndrome	Mutation in NLRP3	-	[63]
Chronic granulomatous disease	Mutation in NADPH oxidase	-	[68]

Table 1: A list of hematological or inflammatory diseases via iPSCs. Mutations were corrected in several diseases to reverse disease phenotype.

Currently many diseases are modeled using iPSCs including neurologic, psychiatric and immunologic diseases **(Table 1)** [61]–[68]. One of the first diseases modeled in this manner were hematological disorders such as thalassemia [69]. Thalassemia is characterized with defective hemoglobin formation caused by mutations in α or β-globin chain which eventually lead severe anemia. Ye et al. reprogrammed fibroblasts of β- thalassemia patient to iPSCs. Differentiated iPSCs were then successfully differentiated into fetal hematopoietic cells that synthesize hemoglobin. However, to generate healthy hematopoietic cells with normal hemoglobin, gene targeting methods should be used. iPSCs were also derived from cells of amniotic fluid and chorionic villus sample which are used for diagnosis of thalassemia.

Thus, iPSCs can be generated, corrected and differentiated into hematopoietic cells during pregnancy for early treatment of thalassemia.

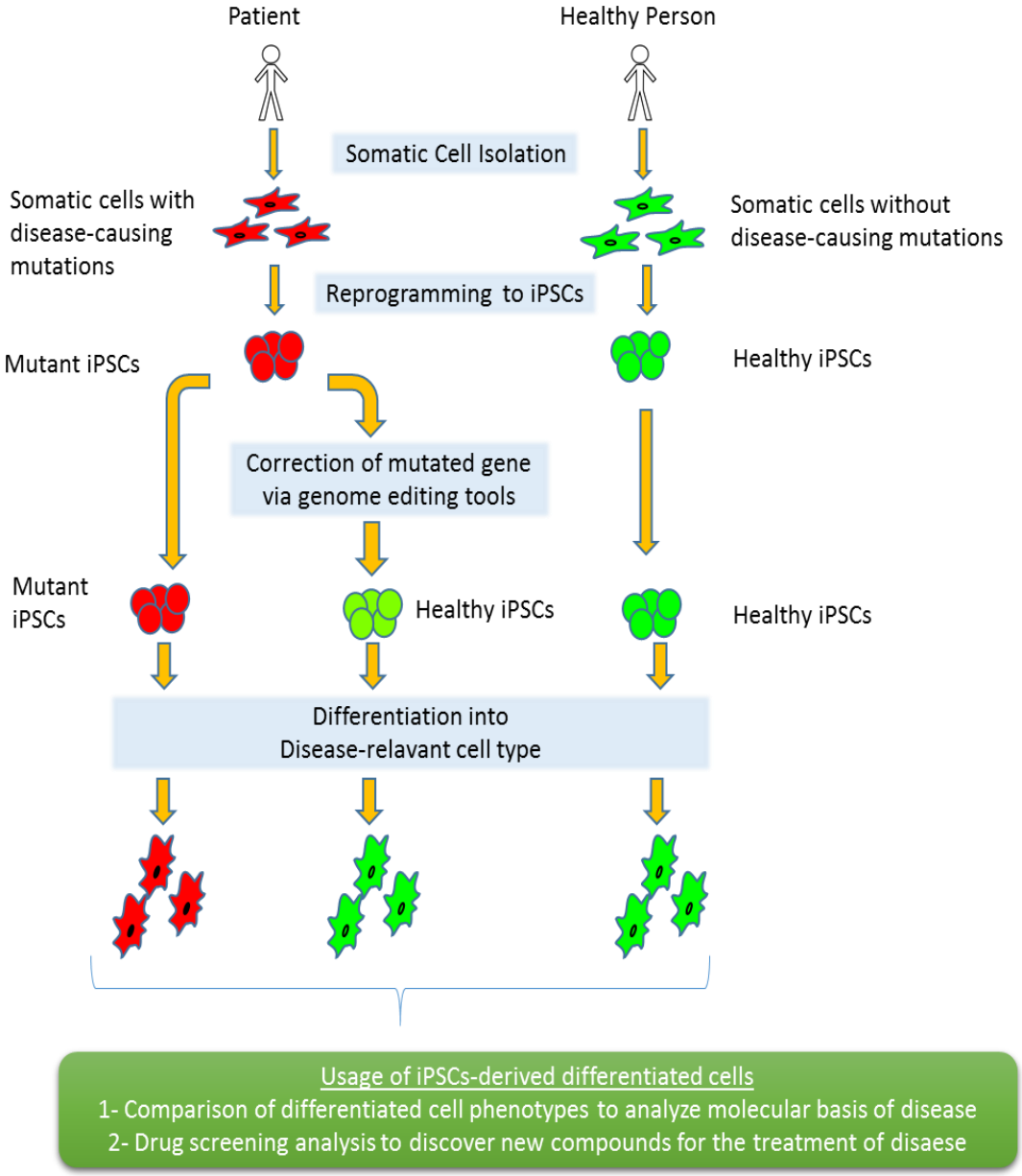


Figure 8: A schematic illustration of disease modeling. Somatic cells are isolated from patient and as a control from healthy person. Mutated cells are reprogrammed to iPSCs. Mutated genes can be targeted in patient iPSCs to recover mutant iPSCs. All iPSCs are differentiated into disease relevant cell types using differentiation protocols. Differentiated cells can be used in both phenotype comparison to evaluate molecular mechanisms of disease and drug screening analysis to discover new compounds that can be used in disease treatment.

Furthermore, inflammatory diseases were modeled such as Gaucher disease, CINCA and Bowl Syndrome [61], [62], [63], [68]. Mutations in the acid β-glucocerebrosidase gene cause the Gaucher disease which is characterized with the presence of lipid filled macrophages [61]. These pathological macrophages are believed as the source of the increased level of inflammatory cytokines in the Gaucher patients. Panicker et al. differentiated iPSCs of patients into macrophages to elucidate abnormalities of macrophages. As a result, the study demonstrated that macrophages of Gaucher patients are abnormal and responsible for increased level of several inflammatory cytokine such as IL-1β, TNF-α and IL-6.

CINCA (Chronic infantile neurologic cutaneous and articular) syndrome is the only auto-inflammatory disease that has been modeled currently [63]. The disorder is caused by NLRP3 mutation which leads abnormal IL-1β secretion in the macrophages. Interesting thing about the syndrome is that NLRP3 mutations are carried as somatic mosaicism. Therefore, separating mutant and wild type macrophages from patients are difficult. Thus generation disease-relevant cell types such as macrophages from mutant iPSCs may provide robust homogenous cell population to study disease mechanism. A first stimulus, such as LPS, in various immune cells including macrophages is needed for inactive pro-IL-1β synthesis. A second stimulus, such as ATP, induces the activation of NLRP3 inflammasome. The inflammasome contains caspase-1 which cleaves the pro-IL-1β to mature IL-1β which is the active and secreted form of IL-1β. Normal monocytes or macrophages have limited or no IL-1β secretion in response to LPS stimulation; however, CINCA patients' cells secreted higher level of IL-1β due to mutant NLRP3 inflammasome which is activated without any second stimulus. To model CINCA disease, Tanaka and colleagues introduced four Yamanaka factors into fibroblasts of CINCA patients using retroviruses. Reprogrammed fibroblasts were then characterized with various assays including gene expression analysis and teratoma formation assay to demonstrate bona fide İPSCs generation. Demethylation of Oct4 and retroviral integration patterns were also confirmed. There were no integration or copy number changes, in any of genes that might affect the function of the NLRP3 inflammasome.

After the characterization of iPSCs, iPSCs were differentiated into macrophages using a murine stromal cell line OP9. iPSCs were cultured on OP9 cell line for 10 days, $CD34^+$ KDR^+ progenitor cells were collected. Macrophages, CD64 positive cells, emerged after $CD34^+$ KDR^+ progenitor cell culture with another OP9 cells for 16 days. Approximately 80% of the progenitor cells were expressed CD14 at the end of the differentiation protocol. To show the disease phenotype in the iPSCs-derived macrophages, wild type and mutant macrophages

were stimulated with LPS. Stimulated mutant macrophages were secreted higher IL-1β than wild type macrophages. Thus, they showed that abnormal function of iPSCs-derived macrophages were determined by NLRP3 mutation. In addition, CINCA patients show a mosaicism for mutant and wild type macrophages. Thus, researchers aimed to simulate a mosaic condition by coculturing mutant and wild type cells. After the stimulation of mutant iPSCs-derived macrophages in separate cultures and in coculture with wild type cells, significantly increased IL-1β secretion was observed in the supernatant of cocultured cells. Thus, they modeled mosaic CINCA disease in the culture conditions and demonstrated that mosaicism affects the disease phenotype by increasing IL-1β secretion. Furthermore, researchers performed a drug screen to treat mutant macrophages by inhibiting NLRP3 activation. Several compounds were used to target upstream or downstream of NLRP3 inflammasome.

One of the challenge in disease modeling is that reprogrammed cell lines may have different biological properties. The differences in cellular activity can generally result from reprogramming methods. Especially, the methods that use random integration of reprogramming vectors into genomic host DNA, may change the cellular gene expression profile which is unrelated with disease [71]. Unrelated phenotype of cells could mislead the conclusion of experiment results. To avoid integration related genetic changes in reprogrammed cells, non-integrating reprogramming methods have been developed with the introduction of plasmids, mRNA or proteins. An additional challenge is incomplete epigenetic reprogramming. Epigenetic state of iPSCs is important to have homogenous differentiated cell population. Improved culture conditions could be used to achieve epigenetic stability among the iPSCs [72].

Since the differentiation ability of iPSCs into three germ layer cells is the key player in disease modeling, protocols that are used for differentiation are crucial. Current methods to differentiate iPSCs into functional cell types mainly rely on growth factors [74]. These methods could be varied by coculturing or enforcing specific gene expressions with viral infection [68], [74]. Even though some cells could be generated efficiently, current protocols generate a mixture of diverse cell types. To confirm the differentiation status of cells, lineage specific reporter or selection genes can be used to monitor terminally differentiated cell types. Still, robust differentiation protocols for many lineages are remained to be discovered.

The main idea in disease modeling is the comparison of phenotype and genotype between healthy and diseased cells *in vitro*. In theory, a genetic mutation or more than one, causes changes in the phenotype and inducing the disease [75]. Therefore, patient-derived and

healthy-derived iPSCs could be a source to analyze differentiated cells with genetic diseases. However, it is a well-known fact that each individual has a unique genetic background. Hence, in reality, healthy and patient iPSCs have various sequence differences in several thousand genes which could alter the protein functions in the cells. Due to the differences in the genetic background that are not related with disease between two cell lines, disease modeling could not be a perfect method to study diseases. On the other hand, recent studies on gene-editing strategies such as CRISPR/Cas9 system, have enabled genetic modifications more efficiently [76]. Therefore, generated iPSC lines could be targeted to modify genes that are related with disease to produce iPSCs with the disease genotype. Genetic manipulations in iPSCs are now promising to generate more reliable iPSC lines for disease modeling.

Huntington disease is one example of genetically corrected iPSCs [77]. Huntington is a progressive brain disorder caused by expanded CAG repeat in *HTT* gene. Patient fibroblasts were corrected by the replacement of expanded CAG repeat with normal repeat utilizing homologous recombination and then differentiated into DARPP-32 positive neurons. Corrected neurons normalized the pathologic signaling including cadherin, TGF-β and caspase activation. Study showed that genetic correction in iPSCs can give rise healthy cell types which can be used in the cell-based therapy.

Amyotrophic lateral sclerosis (ALS) is a fatal disorder that is characterized with motor neuron death. Various mutations can cause the ALS. In one study, patient fibroblasts with SOD1 mutation were reprogrammed into iPSCs [78]. iPSCs then differentiated into motor neurons which exhibit survival deficit, mitochondrial and ER stress. The correction of *SOD1* gene mutation in iPSCs rescued differentiated neuron phenotypes.

The hematologic disease, β-thalassemia, was also modeled and corrected [69], [70]. Deficient β-globins, were corrected with homologous recombination in patient iPSCs. Corrected iPSCs were differentiated into hematopoietic progenitors. Transplantation of progenitors showed improved hemoglobin synthesis in SCID mice. Thus, iPSCs can be derived from patients with hematologic disorders as well as other disorders and iPSCs can be corrected with genome editing methods.

1.2.5 Differentiation Of iPSCs Into Macrophages

Hematopoietic stem cells (HSCs) are the main blood supplier in the mammalians. Whole blood cells including lymphoid, myeloid and erythroid lineage cells are differentiated from HSCs in a strictly controlled manner. Unfortunately, long-term culture of HSCs is not available and HSCs usage is limited to bone marrow transplantation. iPSCs are promising tools to generate HSCs and any blood cell type in vitro [79]. Various protocols have been developed to differentiate iPSCs into different hematopoietic cell types including red blood cells, monocytes, dendritic cells, megakaryocytes and lymphocytes [73], [80]–[82]. In some protocols, iPSCs are differentiated into HSCs and HSCs then differentiated into terminal blood cell types [83], [84]. On the other hand, many studies aim direct generation of terminally differentiated hematopoietic cells from iPSCs without HSCs generation [73].

In this thesis, disease modeling of FMF is aimed by using iPSCs. Since macrophages are one of the primarily affected cells in FMF disease, protocols for iPSCs differentiation into macrophages will be introduced more extensively. Other cell types such as neutrophils can also generated from FMF iPSCs, however, phenotype of generated iPSCs-derived macrophages are enough to show that disease is modeled. Three main methods have been developed to generate macrophages which are embryoid body formation, coculture and viral infection for enforced gene expression. Each technique has advantages and disadvantages as discussed below.

1.2.5.1 Embryoid Body Formation

Culturing of iPSCs or ESCs in a low-attachment plate yields sphere-like structures which are called embryoid bodies (EB). Embryoid bodies consist of randomly differentiated cells and are considered as a 3D culture system. In the normal culture medium, cells in the embryoid body differentiate spontaneously into three germ layer cells [85]. Even though the spontaneous differentiation of EBs is a useful tool to evaluate pluripotency of iPSCs, it is not an efficient method to produce differentiated cell populations. Therefore, EBs are generally transferred into normal attachment plates and cultured in specific medium which contains growth factors.

Generally, generation of macrophage methods consist of 3 steps [73] [86]. In first step embryoid bodies are formed. In second step, EBs are differentiated into monocytes. In last step, monocytes are differentiated into macrophages. At the beginning, iPSCs are seeded in low-attachment plates. Since iPSCs cannot attach to plate as usual, they begin to fold on themselves and form embryoid bodies. Formation of EBs takes one to two days. However, to obtain further differentiated cells, EBs are cultured in low attachment plates for 4 days. On day 4, EBs are manually collected under a stereo microscope and transferred into a normal attachment plate. It is suggested that bigger EBs could produce monocytes more efficiently [86]. Therefore, larger EBs are collected to continue onto the next step. Medium is another crucial factor during differentiation. For monocyte production, EBs are cultured in normal attachment plate in DMEM medium which includes serum and growth factors such as interleukin-3 (IL-3) and macrophage colony stimulating factor (M-CSF). Production of monocytes from EBs takes two to three weeks. Monocytes are released from EBs and can be collected in medium. Collected monocytes are then cultured in DMEM medium in high concentration of M-CSF. In one or two weeks macrophages are generated.

EB formation is a robust protocol to generate macrophages. Production of monocytes continue up to 2 months which allows to collect increased number of monocytes. Also, most of the differentiated cells are monocytes or macrophages, therefore a sorting step is not necessary.

1.2.5.2 Coculture Of iPSCs With Stromal Cells

Coculture of iPSCs with stromal cell lines is another method to generate macrophages [68], [73]. Stromal cells which are derived from bone marrow have ability to differentiate iPSCs into HSC-like cells. These HSC-like cells then can be further differentiated into macrophages. However, coculture of iPSCs leads to the emergence of more than one cell type which in turn requires a sorting step. Flow cytometric sorting enable the isolation of purified HSC-like cells that are ready to differentiate into more specific hematopoietic cells. Sorted HSC-like cells are cultured in a medium including M-CSF (Macrophage colony stimulating factor) to produce macrophages.

Coculture methods generally have three steps [87]. In firs step iPSCs are differentiated into HSC-like cells by coculturing with bone marrow stromal cell lines. In the following step, HSC-like cells are sorted and cultured in a medium with cytokine for proliferation. In third step, expanded and gradually differentiated HSC-like cells are cultured with different cytokines to

generate macrophages. OP9 cell line which is a mouse bone marrow stromal cell line is widely used in coculture for macrophage production. OP9 cells are cultured for 2 weeks to generate confluent cell monolayers. iPSCs are then passaged onto OP9 cells. Coculture takes 10 days. Following to coculture, cells are collected and sorted with proper HSC markers which are generally $CD34^+$ and $CD45^+$. Sorted cells are cultured in a normal plate in a medium with GM-CSF. GM-CSF leads both expansion and differentiation of cells. Therefore, after about 8 days of GM-CSF culture, cells become expanded and more differentiated towards to myeloid lineage. In step three, cells are cultured in a medium with M-CSF which ultimately differentiate cells into macrophages.

1.2.5.3 Viral Infection Of iPSCs

As in reprogramming, specific gene expression enforcement is a method to generate macrophages. Apart from reprogramming, iPSCs could be infected with virus that encodes macrophage specific genes. Enforced macrophage-specific gene expression in iPSCs may lead the macrophage generation.

In a study, mouse fibroblasts are transdifferentiated into macrophages [74]. Basically, fibroblasts are infected with virus that encodes PU.1 and C/EBP-α. Since PU.1 and C/EBP-α upstream genes that control the macrophage related genes, fibroblasts are converted into functional macrophages. Even though this system has not been applied to iPSCs yet, enforced expression of specific genes could succeed the differentiation of iPSCs into macrophages.

CHAPTER 2

Materials And Methods

iPSCs can be generated from various cell type including peripheral blood mononuclear cells (PBMCs) and fibroblasts. In this thesis, FMF Patient and Parent iPSCs were generated from skin fibroblasts. Healthy iPSCs on the other hand were generated using PBMCs. All cell culture steps were done in a laminar flow cabinet. And all reagents were sterile [88].

2.1 Isolation, Culture And Reprogramming Of Cells

2.1.1 Primary Cell Culture Of Patient And Parent Fibroblast

Source of fibroblasts were skin punch biopsies a FMF patient and his parent **(Appendix 2)**. For both cell lines the same protocols were followed. Skin punch biopsies were obtained by a physician (A. Gul) under a protocol approved by the relevant Institutional Review Board. The biopsy punch were kept in fibroblast medium (D10, **Table 2**) on ice during transportation to laboratory.

500 ml DMEM (1x, Basic), (Ca,Mg free) (+Pyruvate)
110 ml FBS (Fetal Bovine Serum)
11 ml L-Glutamine
11 ml Penicillin/Streptomycin

Table 2: Components of D10 medium. All components are mixed at indicated volumes then filtered through 0.22 µm filters.

Biopsies were washed twice with 10 ml DPBS (without calcium and magnesium). Biopsies were then transferred to a 6-well plate using sterile forceps. In order to make small pieces, biopsies were cut into 0.5-1 mm pieces utilizing sterile razor blades. Biopsies were then placed under a cover slip and cultured in one well of six-well plate in 2 ml D10 medium for three days in an incubator at 37°C, 5% CO_2 . Three days later, medium was removed and

fresh D10 medium was added. Cells were checked daily for growth. Medium was changed every other day until fibroblast growth was observed.

It took about 3 weeks to establish cell lines. When cells reached the confluency (90% of the culture area), medium was removed and cells were washed once with DPBS. Passaging was performed in 0.05% trypsin/EDTA solution. Cells with trypsin/EDTA solution were incubated at 37°C for 5-7 minutes. When cells become unattached, fresh D10 was added directly into wells to inactivate the trypsin solution. Cells were passaged at a ratio of 1:3 to new plates.

To generate cell stocks, fibroblasts were freezed in D10 freezing medium (**Table 3**). Prior to use, freezing medium were filtered through a 0.22 µm DMSO-safe filter. Cells were washed once with PBS and incubated at 37°C for 5 min in trypsin/EDTA solution. Fresh medium was added into wells to inactivate the trypsin as 2 fold volume of trypsin/EDTA solution. Cells were collected to falcon tubes and centrifuged at 1200 rpm for 5 minutes. Supernatant was aspirated and cells were resuspended in 2 ml freezing medium per well. Cells were aliquoted into 2 labeled cryovials as 1 ml. Cryovials were placed into -80°C freezer. Next day, vials were moved into the vapor phase of a liquid nitrogen.

40% D10 medium
10% DMSO
50% FBS

Table 3: Components of D10 freezing medium. All components are mixed at indicated volumes then filtered through DMSO-safe 0.22 µm filters.

Cells were thawed whenever they were needed by following steps: Cryovial was taken from liquid nitrogen and was put quickly in a 37°C water bath by shaking gently. When cells were thawed completely, 1 ml D10 medium was added into cryovial and combined with 4 ml D10 medium in a 15 ml Falcon tube. After centrifugation at 1200 rpm for 5 minutes, supernatant was aspirated. Cell pellet were resuspended in D10 medium as required volumes. Homogenous cells were seeded into wells. Next day fibroblasts should be seen as attached to plate.

2.1.2 Peripheral Blood Mononuclear Cell (PBMC) Isolation

To generate episomal vector introduced-iPSCs from PBMCs, peripheral blood was taken from a healthy person and mononuclear cells were isolated by using Ficoll-Paque. 10 ml blood was diluted with PBS as 1:1 ratio. 5 ml Ficoll-Paque was added into empty 15 ml Falcon tube and then 10 ml blood/PBS solution was slowly added on the top of 5 ml Ficoll-Paque. Blood and ficoll were not mixed into each other. Tubes were centrifuged at 400 x g for 30 min at 18°C with slow acceleration. Serum, which was at the top of the tube was aspirated. Buffy coat (off-white layer) that contains PBMCs were collected. Isolated PBMCs were washed for twice with PBS. Cells were then suspended in H3000 medium with cytokines (100 ng/ml IL-6, 300 ng/ml SCF, 300 ng/ml TPO, 300 ng/ml Flt3 ligand, 10 ng/ml IL-3) and seeded as 3 x 10^6 cells into a well of 6-well plate. PBMCs were incubated at 37°C, 5% CO_2, for 6 days. After 5 days of PBMC isolation and culture, mito-MEFs were thawed and seeded as 2 x 10^5 cells into one well of a 6-well plate. Cells were then reprogrammed as explained below.

2.1.3 Mitotically Inactivated Mouse Embryonic Fibroblast (mito-MEF) Preparation

Mito-MEFs are required to culture human ESCs and iPSCs in human embryonic stem cell (hESC) medium since they keep the cells at undifferentiated stage. It is started to be used from the second week of reprogramming and during iPSCs cultures. Therefore, it is essential to prepare mitotically inactivated mouse embryonic fibroblasts.

To isolate mouse embryonic fibroblasts (MEFs), a pregnant mouse was sacrificed at 13,5 d.p.c (days post-coitum) by cervical dislocation. Uterine horns were dissected out, rinsed in 70% ethanol and placed into a falcon tube containing PBS without Ca^{2+} and Mg^{2+}. Note that the following steps were performed in tissue culture hood under aseptic conditions. Before fibroblasts isolation, 10 cm^2 petri dishes were coated with 0.1% gelatin for at least 10 minutes at room temperature. Embryos were placed in petri dishes that contains PBSs to separate them form placenta and embryonic sac. Separated embryos were then dissected to discard head and red organs (for instance, liver). At the end, remaining embryos were minced utilizing razor blades until it becomes possible to pipette. Trypsin/EDTA (0.05%) was added onto tissues, approximately 1 ml trypsin for 1 embryo. Tissue/trypsin mixture was collected into falcon and incubated at 37°C for 15 minutes. Cells were mixed by pipetting up and down at each 5 minutes of incubation. To inactivate trypsin, equal volume of D10 medium was

added. Cells were then incubated at 1500 rpm for 5 minutes. Approximately, 2 embryos were cultured in one $10cm^2$ petri dish which contains 12 ml D10 medium. Thus, supernatant was removed and cells were resuspended in proper volume of D10 medium. Gelatin was aspirated and cells were seeded. MEFs were cultured till they become confluent.

To inactivate MEFs, cultured MEFs were passaged twice using trypsin/EDTA with 1:4 and 1:5 ratio. When the cells reach approximately 80% confluency, medium was removed from MEFs and cells were washed with PBS. Washed cells were incubated in 20 ml D10 medium containing 10 µg/ml mitomycin C in 37°C for 2 hours. After 2 hours of incubation, medium was removed, MEFs were then washed twice with PBS and trypsinized. Lifted cells were counted and freezed in D10 freezing medium (40% D10, 50%FBS and 10%DMSO) at desired cell numbers. After this treatment, MEFs were named as mito-MEFs.

To plate mito-MEFs for iPSCs culture, wells of a 6-well plate were coated with 0.1% gelatin for 10 minutes at room temperature. A cryovial that has 1.2×10^6 mito-MEFs was taken from liquid nitrogen and thawed quickly in 37°C water bath. Cells were then combined with 4 ml D10 medium and centrifuged at 1200 rpm for 5 minutes. After centrifugation, supernatant were discarded and cells suspended in 12 ml D10 medium. Gelatin in the wells were aspirated and cell suspension added into each well as 2 ml. Mito-MEFs were cultured in a 37°C, 5% CO_2 incubator. At the end, each well had 2×10^5 cells.

Even though the technique of mito-MEFs thawing is similar to normal fibroblast thawing, it should be kept in mind that timing of thawing is quite important for mito-MEFs. Mito-MEFs have very limited life span and they could support iPSCs as undifferentiated cells up to 7-8 days. This means iPSCs should be passaged every week onto fresh mito-MEFs. For instance, to passage fibroblasts on day 7 of reprogramming, mito-MEFs should be thawed one day before (on day 6). It is same for regular passage of iPSCs which mito-MEFs should be thawed one day prior to passage.

2.1.4 Reprogramming Of Fibroblasts By Introducing Episomal Vectors With Electroporation

Integration-free iPSCs were generated using episomal vectors which encodes reprogramming factors Oct4, Sox2, Lin28, L-Myc, Klf4 and also shp53 **(Figure 9)** [89]. In order to introduce episomal vectors an electroporation system, “Neon Transfection System”, was utilized. Prior to starting electroporation, cells (both fibroblasts and PBMCs) were cultured as explained previously to obtain enough cell number.

Neon transfection system was prepared by filling electroporation cuvette with 3 ml E2 buffer. For one electroporation, each of the three episomal reprogramming plasmids (pCXLE-hOCT3/4-shp53-F, pCXLE-hUL, pCXLE-hSK **(Table 4)** were combined as 1.2 µg in 120 µl resuspension buffer R using a 1.5 ml micro centrifuge (Eppendorf) tube. Final plasmid concentration would be 3.6 µg plasmid in 120 µl R buffer.

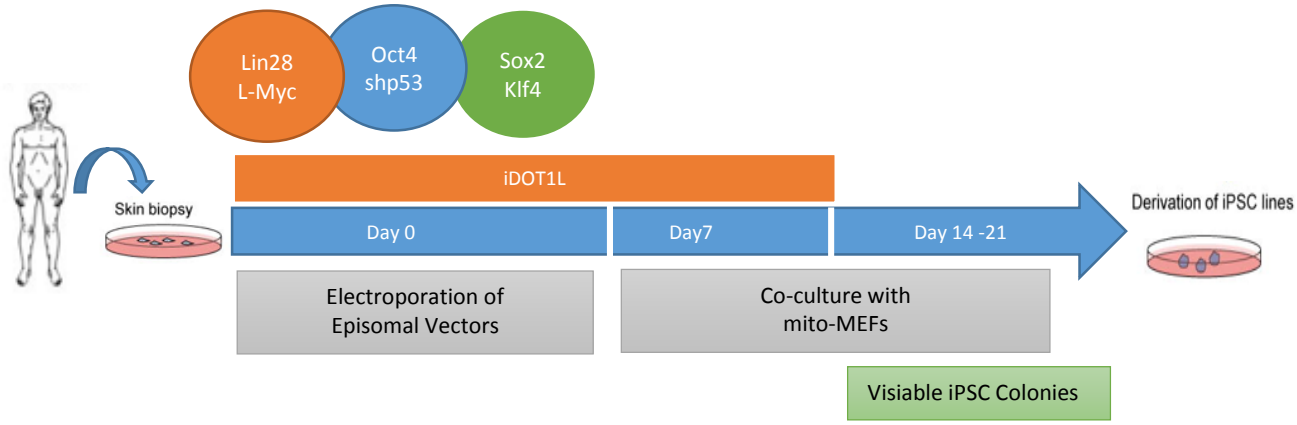


Figure 9: Flow chart of episomal reprogramming. Episomal vectors are introduced to somatic cells. After 1 week of electroporation, cells are cultured on mito-MEFs. Aproximatelly in 2 weeks iPSCs colonies become visiable. To increase reprogramming efficiency, Dot1L is used for 2 weeks at the begining.

Confluent cells were detached with trypsin/EDTA and counted. $3x10^5$ cells are required for each electroporation. Cells were then centrifuged at 1200 rpm for 5 minutes and supernatant was aspirated. Cells were resuspended in 120 µl R buffer/plasmid solution and mixed. 100 µl of R buffer, plasmids and cells mixture were taken into 100 µl tip of Neon transfection kit. Tip was inserted into cuvette. Parameters were set as 1400V, 20ms, 2 pulse and electroporation was performed. After electroporation is completed which takes a few seconds, solution in the tip was transferred into one well of 6-well plate that contains 2ml D10 medium without

antibiotics. It is important to note that electroporation could make the cells sensitive to antibiotics. Therefore for first day of electroporation, cells were cultured in D10 without antibiotics. Cells were cultured in 37°C, 5% CO_2 incubator. This was considered as day 0 of reprogramming.

Plasmid Name	Addgene Plasmid Number
pCXLE-hOct3/4-shp53	27077p
pCXLE-hSK	27078
pCXLE-hUL	27080
pCXWB-EBNA1	37624

Table 4: Episomal reprogramming vectors and addgene plasmid numbers.

Next day (on day 1 of reprogramming), D10 medium was changed with fresh D10 that contains 1% penicillin and streptomycin antibiotics. In order to increase reprogramming efficiency, 3 µM Dot1L inhibitor (EPZ004777, iDot1L) was also added during first 2 week of reprogramming [90]. On day 7, cells were passaged onto mitotically inactivated MEFs (mito-MEFs) in hESC medium **(Table 5)**. When colonies were formed and become to proper size, passaged either manually or enzymatically.

DMEM/F12 (Basal Medium)
20% KOSR (Knock-out Serum Replacement)
10 ng/ml bFGF
0.1 mM β-mercaptoethanol
1% Non-Essential Amino acids (MEM-NEAA)
1% L-Glutamine
1% Penicillin/Streptomycin

Table 5: Components of hESC medium. All components are mixed at indicated volumes then filtered through 0.22 µm filters.

2.1.5 Reprogramming Of Peripheral Blood Mononuclear Cell To iPSCs

Electroporation which is explained in section “Reprogramming of Fibroblasts” with few modifications was performed using cultured PBMCs from section “Peripheral Blood Mononuclear Cell Isolation”. To increase efficiency of episomal vectors, one additional vector was also introduced into PBMCs which is pCXWB-EBNA1. Since cell type is different than fibroblast, electroporation setting was adjusted as 2150V, 20ms, 1 pulse. At the end of the electroporation, PMBCs were seeded onto mito-MEFs in H3000 medium containing cytokines. On day 8, 10 and 12, fresh 1.5 ml mTeSR1 medium was added into each well without aspirating any of medium. Medium was fully changed with mTeSR1 on day 14. When colonies were formed and become to proper size, they were passaged either manually or enzymatically.

2.1.6 Manual And Enzymatic Passaging Of iPSC

When iPSCs become large enough, they were passaged either manually or enzymatically. Manual passaging is rather used for early passages of iPSCs when a few colonies are found in a well. However, if colony number is increased in further passages, enzymatic passage is preferred. Mito-MEFs should be thawed and seeded as 2 x 10^5 cells per well of a 6-well plate one day prior to iPSCs passaging.

To passage iPSCs manually; 1 ml hESC medium was added 2 hours before passaging. This reduces passaging stress and keeps iPSCs in a better condition. Undifferentiated iPSCs colonies were marked under an inverted microscope. With the help of a stereo microscope in the laminar flow hood, marked colonies were divided into small pieces using a 10 µl pipette tip. Divided colony pieces were transferred into a new well which were seeded with mito-MEFs in hESC medium. Medium was changed daily with fresh hESC medium till the colonies become large enough to be passaged again. This takes 5-7 days.

To passage iPSCs enzymatically; differentiated colonies were marked under an inverted microscope. Marked colonies were then removed using a 10 µl pipette tip under a stereo microscope in the laminar flow hood. Medium was aspirated and cells were washed once with DMEM/F12. 1 ml of Collagenase IV (1 mg/ml) was added into each well and cells were incubated at 37°C for 5 minutes. Collagenase IV was aspirated. To remove remaining collagenase, cells were washed once with DMEM/F12. 1 ml DMEM/F12 was added into each

well and cells were scraped utilizing a cell lifter. Detached cells were collected to a falcon tube and centrifuged at 1000 rpm for 2 minutes. Supernatant was discarded and pellet was carefully resuspended in fresh hESC medium. It is important to keep iPSCs as colonies. Therefore, breaking down of colonies into single cells should be avoided during passaging. Resuspended iPSC colonies seeded onto mito-MEFs at a ratio of 1:2 or 1:5.

2.2 Characterization Of iPSCs

2.2.1 Gene Expression Analysis

Generated iPSCs were subjected to qPCR for gene expression analysis. Both FMF patient, parent and healthy iPSCs were collected from cell culture with collagenase IV. RNA of collected cells were isolated utilizing a commercial RNA isolation kit according to manufacturer's manual (Qiagene). cDNA was synthesized using 1 µg RNA for each sample. Produced cDNA samples from mRNA with random hexamers were used in qPCR to evaluate Oct4, Sox2 and Nanog gene expressions of cell lines. Primer sequences and PCR setting is shown in **Table 6** and **Table 7**, respectively.

Label	Sequence
hOct4 Forward	CCTCACTTCACTGCAACTGTA
hOct4 Reverse	CAGGTTTTCTTTCCCTACGT
hSox2 Forward	CCCAGCAGACTTCACATGT
hSox2 Reverse	CCTCCCATTTCCCTCGTTTT
hNanog Forward	TGATTTGTGGGCCTGAAGAAA
hNanog Reverse	TGGTGGTAGGAAGAGTAAAG

Table 6: Primer list used in gene expression analysis.

	Temperature	Duration	Cycle Number
Cycle 1:	95°C	3:00 minutes	1
Cycle 2:	95°C 60°C 72°C	10 seconds 30 seconds 30 seconds	40
Cycle 3:	72°C	5:00 minutes	1

Table 7: qPCR settings for gene expression analysis of pluripotent genes.

2.2.2 Immunofluorescence Staining

iPSCs express pluripotency related genes as well as proteins such as Oct4, Nanog and SSEA-4 (another pluripotency related protein, Stage Specific Embryonic Antigen-4). Therefore I stained iPSCs colonies to demonstrate the presence of pluripotency markers at protein level.

Prior to immunofluorescence staining (IF staining), iPSCs were cultured in 12-well plates containing 12 mm autoclaved cover slips which are coated with matrigel. After 3 or 4 days, colonies were become to appropriate size for staining. As first step, cells were fixed in 4% PFA (paraformaldehyde) for 30 minutes at room temperature. After washing fixed cells, 0.2% Triton-X solution was added to permeabilize cells for 30 minutes. To block non-specific binding of primary antibodies, 3% BSA (Bovine serum albumin) and 5% donkey serum were added after permeabilization step. 2 hours later, BSA and donkey serum were removed and each cover slip was treated with one specific primary antibody such as Oct4, Nanog and SSEA-4 **(Table 8).** Antibodies and cells were then incubated at +4°C for overnight. Next day, cells were washed for at least 5 times to remove all non-binding primary antibodies. Oct4 and Nanog primary antibodies are not conjugated with fluorophore in contrast to SSEA-4 which is conjugated. Therefore, washed cells that stained with fluorophore conjugated primary antibodies (SSEA4) were directly mounted with 1:10,000-diluted Hoechst 33342. Other cells that stained with fluorophore unconjugated primary antibodies (Oct4 and Nanog) were

treated with fluorophore conjugated secondary antibody. After 3 hours incubation of cells with secondary antibodies at +4°C, cells were washed with PBS and then mounted in Hoechst 33342. By using a confocal microscope, images were taken.

Target	Vendor	Primary Antibodies	Secondary Antibodies
Oct4	Abcam Ab19857	Rabbit IgG, diluted 1:100 in blocking buffer*	Alexa Fluor 488 Anti-rabbit diluted in 1:300 in blocking buffer
Nanog	Abcam Ab21624	Rabbit IgG, diluted 1:100 in blocking buffer	Alexa Fluor 488 Anti-rabbit diluted in 1:300 in blocking buffer
SSEA4	BD 560218	Mouse IgG3k, dilute 1:100 in blocking buffer Alexa fluor 555	-

Table 8: Antibodies used in immunofluorescence staining. *Blocking buffer was prepared with 5% Donkey Serum and 3% Bovine Serum Albumin in PBS.

2.2.3 Analysis Of Episomal Vector Integration

Since both fibroblasts and PBMCs were reprogrammed with episomal vectors, reprogrammed cells should be integration-free. However, a chance of vector integration into genomic DNA is still exist [56]. To eliminate this possibility, PCR-based method was utilized. Because of all episomal vectors includes EBNA sequence, in the case of integration of any vector into genomic DNA, a band would be appeared in the agarose gel after PCR amplification. Genomic DNA from all cell lines were isolated using commercial genomic DNA isolation kit as indicated by manufacturer (Qiagen). 50 ng genomic DNA were utilized per PCR reaction with EBNA and GAPDH primers. Primer sequences and PCR settings are shown in **Table 9** and **Table 10**, respectively.

Label	Sequence
EBNA Forward Primer	GTGGTTTGGAAAGCATCGTGGTCA
EBNA Reverse Primer	ATAGGTGGAAACCAGGGAGGCAAA
GAPDH Forward Primer	GAA GGC TGG GGC TCA TTT
GAPDH Reverse Primer	CAGGAGGCATTGCTGATGAT

Table 9: Primer list used in gene integration analysis.

	Temperature	Duration	Cycle Number
Cycle 1:	95°C	3:30 minutes	1
Cycle 2:	95°C 65°C 72°C	10 seconds 30 seconds 30 seconds	40
Cycle 3:	72°C	5:00 minutes	1

Table 10: PCR settings for gene integration analysis.

2.2.4 Karyotype Analysis of iPSCs

Karyotype indicates the number and appearance of chromosomes of a cell. Long term culture of cells is critical for the karyotype of cultured cells. Since iPSCs are cultured for long term, culturing conditions or even reprogramming process may lead to chromosomal abnormalities. To show that generated cell lines do not have any chromosomal abnormality, all three iPSC lines are prepared for karyotype analysis. Cells were sent to a genetic center (Nesiller Genetics). Cell cycle was arrested at metaphase and G-banding was performed.

Giemsa staining was performed for G-banding that heterochromatic regions [91]. Thus chromatin structure and numbers were analyzed.

2.2.5 Teratoma Formation

To demonstrate pluripotent abilities of generated iPSCs lines, teratoma formation assay was performed [92]. Confluent undifferentiated iPSCs colonies from 6 well of a 6-well plate were treated with collagenase IV for 5 minutes at 37°C. After 5 minutes, collagenase IV was removed and cells were washed with DMEM/F12. After adding DMEM/F12 into each wells, cells were lifted using a cell lifter (Corning). Collected iPSCs were centrifuged at 1000 rpm for 2 minutes. Supernatant was removed and pellet was combined with cold Matrigel and D10 (fibroblast medium) at 1:1 ratio (120 µl matrigel, 120 µl D10). Using a 1 ml syringe and 21 G needle, 100 µl matrigel-D10-iPSCs mixture was intramuscularly injected into one leg of SCID mice. Remaining 100 µl cell mixture was injected to other leg of mouse. Mice were monitored for 6 or 10 weeks for teratoma formation. When Teratoma was observed, mice were sacrificed via an IACUC-approved method. Teratoma was dissected as one piece. Teratoma mass was washed twice with PBS and fixed in 10% Formalin for 48 hours at room temperature. Teratoma samples were then sent to a histopathology department for hematoxylin and eosin staining. A pathologist (Prof. Dr. Arzu Ruacan) confirmed the patterns of three germ layer structures in the Teratoma staining.

2.3 Differentiation Of iPSCs Into Macrophages

To differentiate iPSCs into macrophages, three different culture protocol were followed [68], [74], [86]. First method is embryoid body (EB) formation. After generation of EB, cells were cultured in a medium including cytokines to generate monocytes and macrophages. Second method is coculturing of iPSCs and OP9 cells. Final protocol is infection of iPSCs with PU.1 and C/EBP-α expressing virus.

2.3.1 Embryoid Body Formation Protocol

Several protocols were performed to efficiently generate macrophages via embryoid body formation [66], [67]. A main protocol was modified step by step to produce monocyte. A flowchart of main macrophage generation protocol through EB formation is shown in **(Figure 10).**

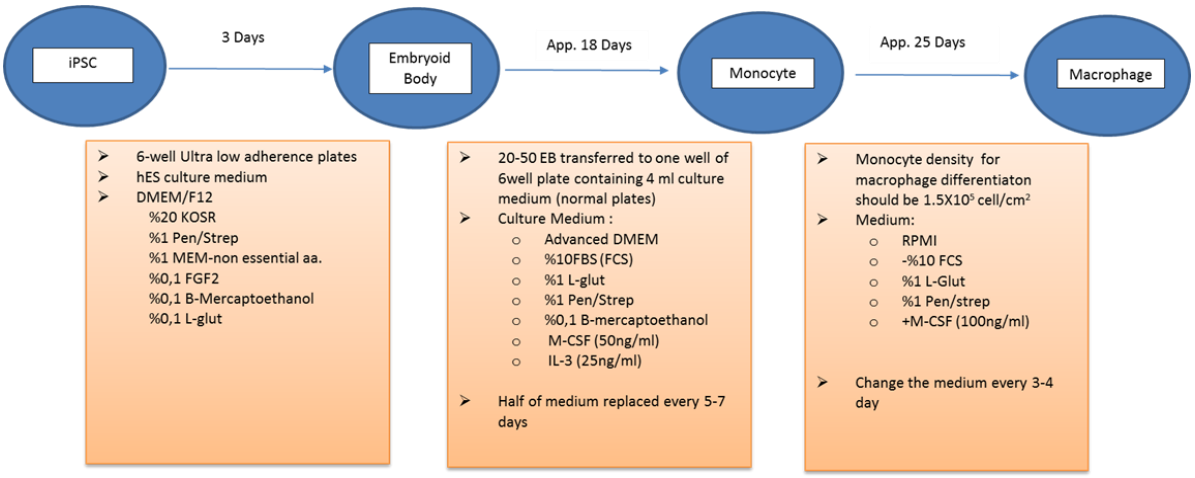


Figure 10: Macrophage differentiation protocol using embryoid body formation. All modificartions for EB formation protocols were derived from illustrated protocol. Adapted from reference [73].

As first step of the differentiation method, iPSCs were collected into falcon tubes using collagenase IV. Collected iPSCs were centrifuged at 1000 rpm for 2 minutes at room temperature. Supernatant was removed and cell pellet was resuspended in normal hESC medium (with 10 ng/ml FGF) and seeded in one well of 6-well low-adherent plate. Plate was either placed into an incubator without shaker or on a shaker, at 100 rpm, in the incubator at 37°C, 5%CO_2. EBs were cultured for 4 days and medium was changed on day 3. On day 4, largest EBs were manually collected under a stereo microscope in a laminar flow hood. Approximately 20-30 EBs were seeded into a 0.1% gelatin coated 6-well plate which contains monocyte differentiation medium (25 ng/ml IL-3, 50 ng/ml M-CSF in D10 medium). Medium was replaced every 3-4 days. After 2 weeks of EB formation, monocytes were expected to be generated and released into medium. Medium was collected to analyze generated cells.

However, protocol used for macrophage differentiation via EB formation was not efficient as expected. Therefore, I modified protocol by changing various steps. Experiment setups with modified steps based on main protocol are explained:

Experiment set 1: At the beginning, I cultured patient iPSCs in normal hESCs medium that contains normal bFGF concentration (10 ng/ml). Cultured iPSCs were then passaged to form EBs at 1:2 ratio. After 3 days, EBs were manually selected and cultured in monocytic differentiation medium as shown in figure.

Experiment set 2: Since attachment of EBs to plate was not efficient, I coated plates with 0.1% gelatin. Remaining steps were same with experiment set 1.

Experiment set 3: PBMC iPSCs were cultured in hESCs medium with 4 fold bFGF concentration. iPSCs were then formed EB for 5 days. Largest EBs were collected manually under a stereo microscope. EBs cultured in 0.1% gelatin coated plates. Remaining steps were same with experiment set 2.

Experiment set 4: Final modification was about the confluency of passaged iPSCs. To generate EBs from iPSCs, 3 well iPSCs of 6-well plate was passaged into 1 well of 6-well low-attachment plate. Thus only starting cell confluency was changed. Remaining steps were same with experiment set 3.

2.3.2 Coculture Of iPSCs And OP9

Coculture of iPSCs with OP9 is second method used in this thesis to generate macrophages **(Figure 11).** Patient-derived iPSCs were cultured in normal hESC medium on mito-MEFs to be expanded. Prior to coculturing of iPSCs and OP9 (a mouse bone marrow stromal cell line), OP9 cells were seeded into 0.1% gelatin coated 6-welll plate as 2 $x10^5$ cells per well in OP medium (10% FBS in α-MEM Medium). OP9 were cultured for 10 days in OP9 medium by changing medium every 3 days. On day 10 of OP9 culture, iPSCs were collected from mito-MEFs using collagenase IV and seeded onto OP9 cells in OP9 differentiation medium **(Table 12)**. Cells were collected on day 7 to analyze gene expression of iPSCs [68].

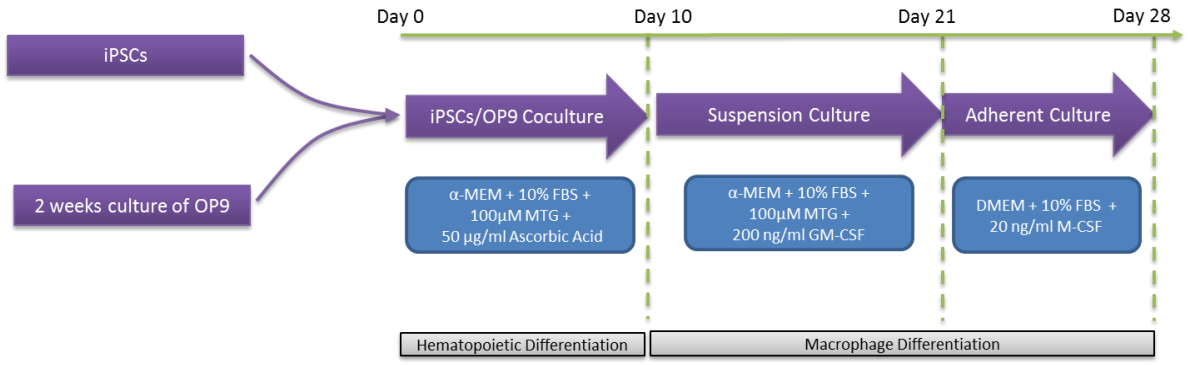


Figure 11: Macrophage differentiation protocol using OP9 coculture. iPSCs were cocultured with OP9 cells for 10 days. After 10 days, cells were cultured as suspension culture in medium with GM-CSF cytokine. For further differentiation, cells were cultured in adherent culture for 7 days in medium with M-CSF cytokine. Adapted from reference [68].

α-MEM Medium
10% FBS
100 μM MTG (Monothioglycerol)
50 μg/ml Ascorbic Acid

Table 12: Components of OP9 differentiation medium. All components are mixed at indicated volumes then filtered through 0.22 μm filters. Note that prior to usage, M-CSF or GM-CSF may added at indicated concentrations.

2.3.3 Staining Cell Surface Antigens For Flow Cytometry

Flow cytometry measures different characteristics of particles flowing in a fluid. Flow cytometry can be used to characterize cells measuring cell size, cytoplasmic granularity, DNA or RNA content, and various intracellular and surface proteins [93].

To evaluate the phenotype of generated cells from iPSCs, I utilized flow cytometry method. Differentiated iPSCs using EB formation and OP9 coculture methods, were stained with fluorophore conjugated antibodies CD45 and CD14. Also, cells were treated with PI (propidium iodide) which shows the cell viability [94].

In EB formation method, attached EBs release monocytes into medium. Therefore, in order to stain EB-derived cells, medium was simply collected into a falcon tube. Similarly, THP1 cells which are monocytic suspension culture were also collected into a falcon tube. On the other hand, attached cells, iPSCs-OP9 coculture system, were treated with trypsin/EDTA solution for 5 minutes at 37°C. When cells were detached, proper OP9 medium was added to inhibit trypsin. Hence, same staining protocol can be followed for all cell types: Cells were washed once with PBS. Washed cells were then resuspended in Flow Buffer (1% BSA in PBS) and 100 µl of cell suspension was put into two falcon tubes. Cells than incubated with CD14 and CD45 antibodies on ice for 30 minutes. Thus, in the one tube, cells were stained with CD45 antibodies and in the other tube cells were stained with CD14. After 30 minutes, cells were washed twice with cold PBS and analyzed in flow cytometer. Furthermore, to evaluate death cells, (PI) propidium iodide was added into CD45 stained cells since CD45 and PI can be detected at different filters. PI was added at 1:20 ratio prior to flow cytometry analysis.

2.3.4 Virus Production

To produce PU.1 and C/EBP-α encoding viruses, I generated retro and lentiviruses. Prior to viral packaging, 2.5 x 10^6 HEK 293T cells were seeded to 10 cm plates. The next day, in the afternoon, HEK cells were transfected with 225 ng VSVG (envelope protein), 2250 ng Gag Pol (pUMVC for retroviruses and 8.2 Delta-VPR for lentiviruses) and 2500 ng viral vector using 20 µl FuGENE 6 Transfection Reagent (Promega) in a total volume of 400 µl serum-free DMEM according to the manufacturer's instructions. 1 day later of transfection, in the morning, the transfection medium was replaced with 8 ml D10 medium. 48 hours after transfection, medium which contains viral particles was collected and stored at 4°C. Fresh

medium was added to transfected 293T cells. Medium was collected 72 hours after transfection. Collected mediums were combined and filtered through a 45 µm filter to remove cells and debris.

2.3.5 Concentration Of Viruses

To precipitate viral particules, 50% PEG-8000 (Sigma) solution was prepared in PBS at 5X concentration. Solution was autoclaved to dissolve and sterilize. 10 ml sterilized 50% PEG solution was added onto 40 ml filtered viral medium. Viral medium and PEG solution was stored overnight at 4°C. The next day, tubes were centrifuged at 2500 rpm for 20 minutes. Supernatant was discarded and a small volume of supernatant was left to not disrupt pellet. Remaining pellet-supernatant mixture was further centrifuged at 1200 rpm for 5 minutes. Supernatant was removed and 300 µl PBS was added to pellet to suspend by pipetting. Collected viral particles were completed to 500 µl adding PBS. Viruses were aliquoted to small volumes and stored at -80°C.

CHAPTER 3

Results

3.1 Fibroblasts And PBMCs Were Isolated

To generate iPSCs from FMF patient, skin biopsy of FMF patient was performed and a fibroblast cell line was established. In additionally, skin fibroblasts from FMF parent were also taken and established as cell line. To compare results, as a control group, a healthy person's peripheral blood mononuclear cells were isolated with Ficoll-Paque and cultured till reprogramming. Morphology of isolated fibroblasts and mononuclear cells is shown in **Figure 12**.

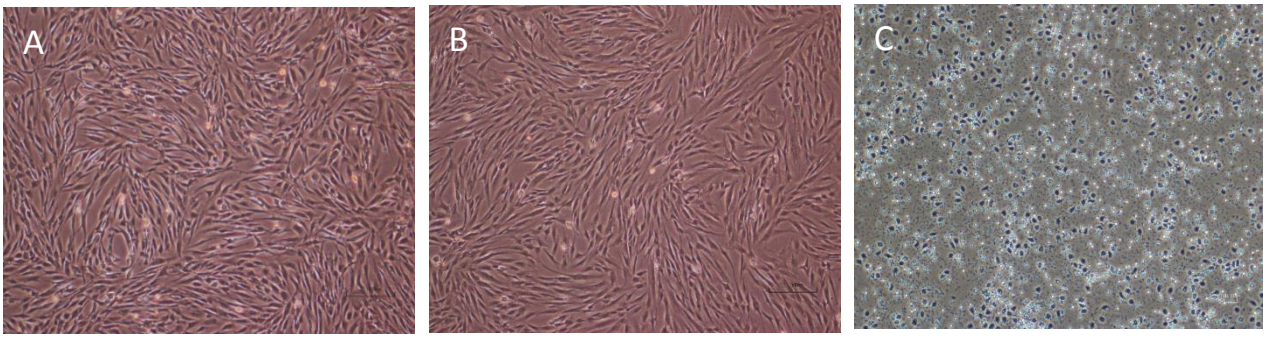


Figure 12: Culture of fibroblasts and PBMCs prior to reprogramming.
A:Patient Fibroblasts, B:Parent Fibroblasts, C:PBMCs

3.2 Patient, Parent and Healthy iPSCs Were Generated With Episomal Vectors

To generate integration-free iPSCs, I introduced the reprogramming factors into fibroblasts and PBMCs using episomal vectors that encode Oct4, Sox2, Klf4, Lin28, L-Myc and sh-p53. I utilized Neon Transfection System for electroporation to enable episomal vector delivery into cells. After 1 week of reprogramming, cells were passaged onto mitomycin inactivated mouse embryo fibroblasts (Mito-MEFs) to support iPSCs formation. After 2 or 3 weeks epithelial-like colonies, similar to human iPSC colonies, were formed which were manually picked and expanded **(Figure 13)**. At the end of the reprogramming procedure, I produced three cell lines derived from a FMF patient, his carrier parent and unrelated healthy donor.

However, formation of colonies are not sufficient to conclude that formed cells are iPSCs. In order to demonstrate that formed colonies are true iPSCs, I further characterized all three cell line with various assays.

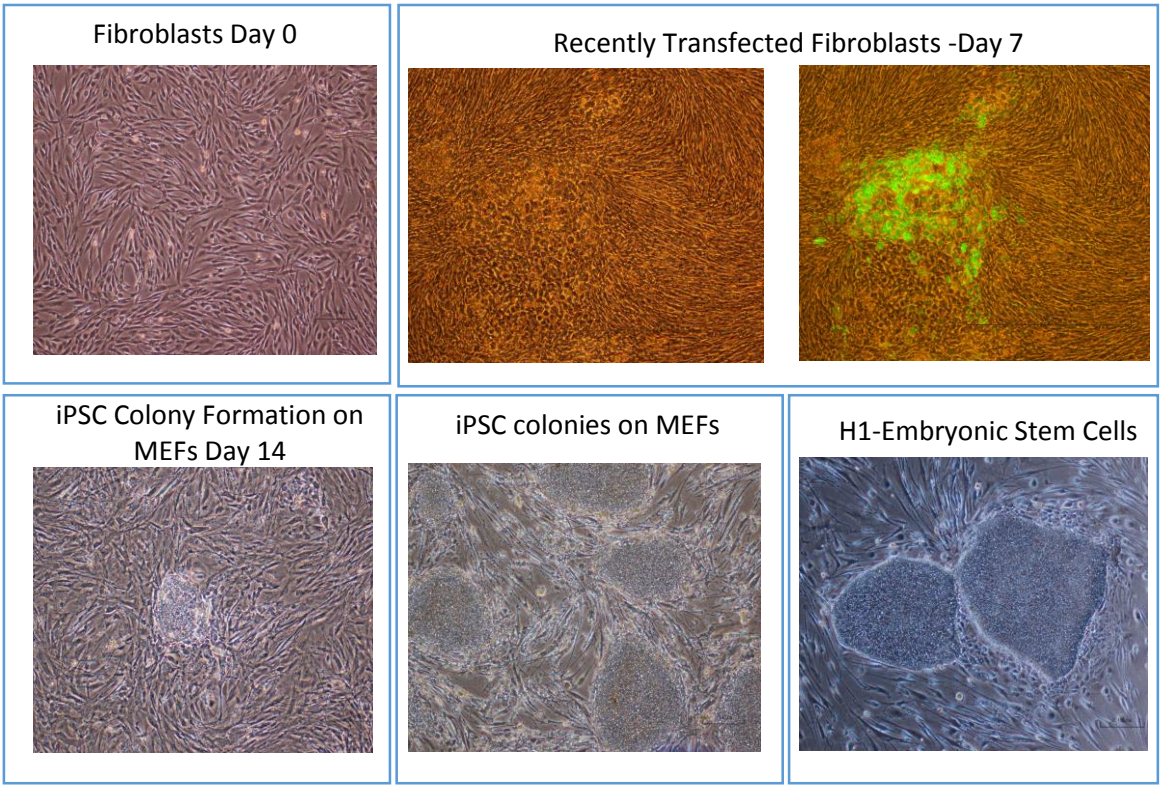


Figure 13: Morphological change patient fibroblasts during reprogramming. On day 0 cells have basic fibroblasts morphology. 7 days after reprogramming, fibroblastic morphology was changed slightly. To show electroporation efficiency, an episomal GFP vector (PCXLE-eGFP) was also introduced with reprogramming vectors (GFP positive cells on Day 7). On day 14, colonies initiated to be formed. Morphology of generated colonies were similar to embryonic stem cells.

3.3 iPSCs Express Pluripotency Genes

Pluripotency genes should be expressed to reprogram differentiated cells into pluripotent stem cells. Pluripotent gene expressions are also needed to maintain iPSCs as undifferentiated cells. To demonstrate that formed colonies are true iPSCs, I performed qPCR using primers for pluripotency genes such as Oct4, Sox2 and Nanog. Relative mRNA levels of samples were measured using relative quantification method which is based on the comparison of a target gene and a housekeeping gene expression levels.

qPCR analysis shows that all three iPSC lines express Oct4, Sox2 and Nanog genes at similar levels with embryonic stem cells **(Figure 14).** However, fibroblasts do not express any gene that is related with pluripotency. Therefore, qPCR results demonstrate the cell lines express marker genes of pluripotency.

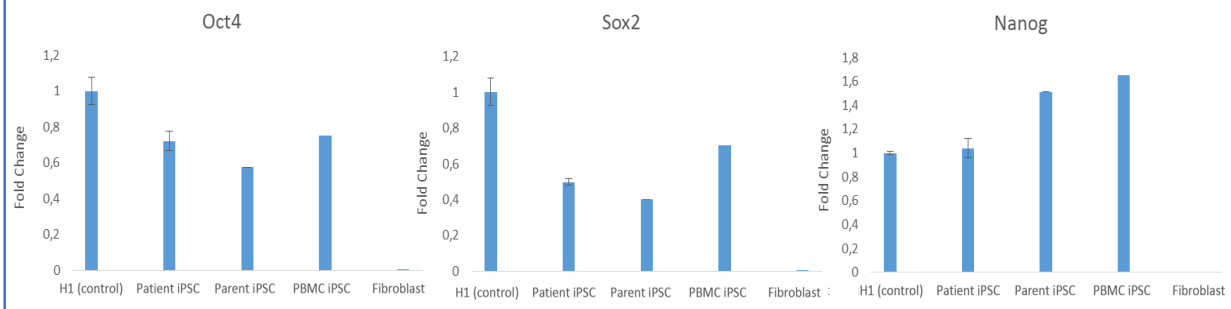


Figure 14: Gene expression Analysis of iPSCs. Three pluripotent genes, Oct4, Sox2 and Nanog, were measured using qPCR.

3.4 iPSCs Express Pluripotency Proteins

Pluripotent gene expression is one of the marks of iPSCs. However, gene transcripts should also be translated into proteins. To analyze the presence of the proteins such as Oct4, Nanog and SSEA-4, I performed immunofluorescence staining of iPSC lines. As it is shown figure, all three cell lines express pluripotency proteins (**Figure 15**, for patient iPSCs; **Figure 16**, for parent iPSCs; **Figure 17,** for PBMC iPSCs). Note that Nanog and Oct4 are nuclear proteins which merge with nuclei staining. However, SSEA4 is a surface (membrane) protein which does not merge with nuclei staining and seem as separated dots around the nuclei. Hence, another marker of pluripotency is observed in generated iPSC lines.

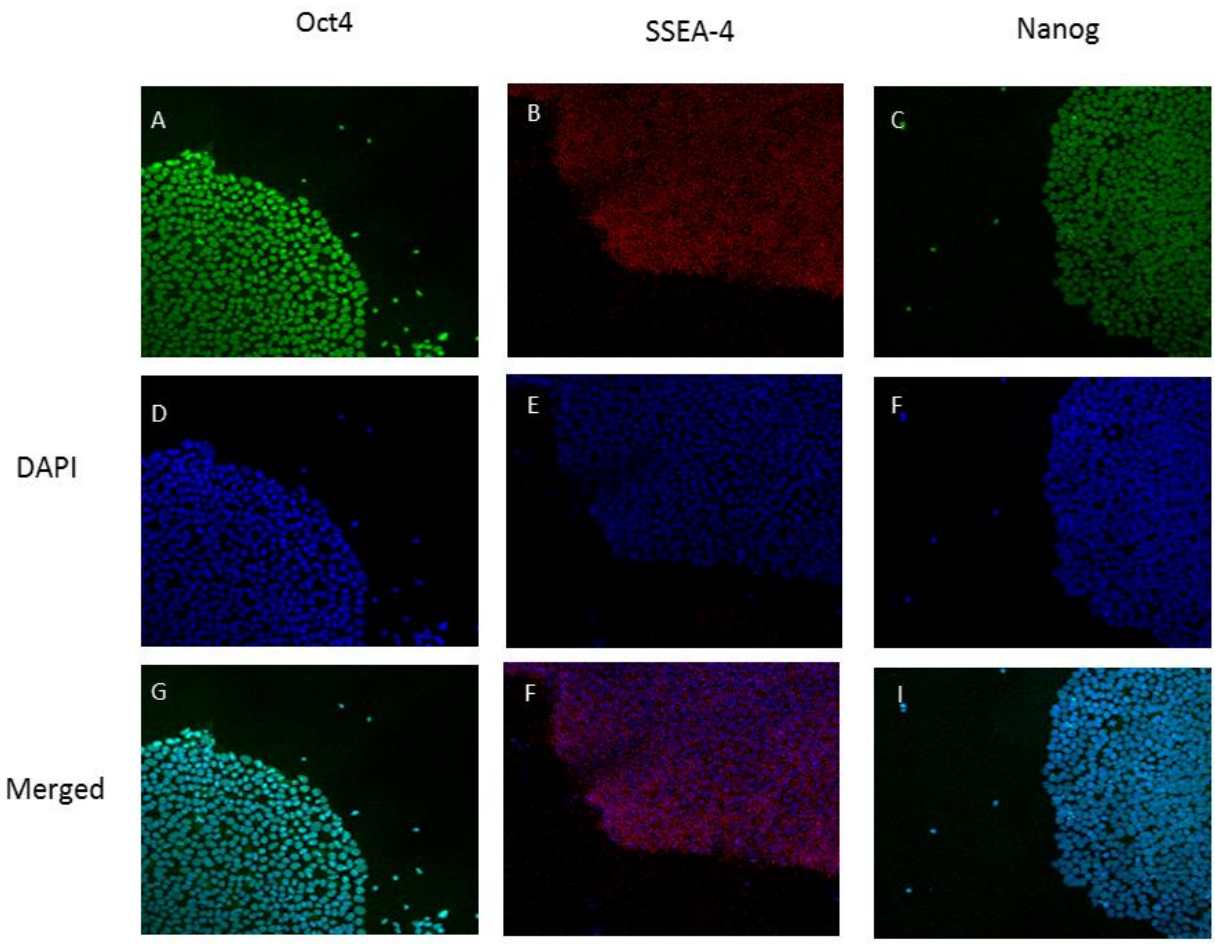


Figure 15: Immunofluorescence staining of Patient iPSCs. Three pluripotency related proteins are stained including Oct4, SSEA4 and Nanog. DAPI is used to indicate nucleus.

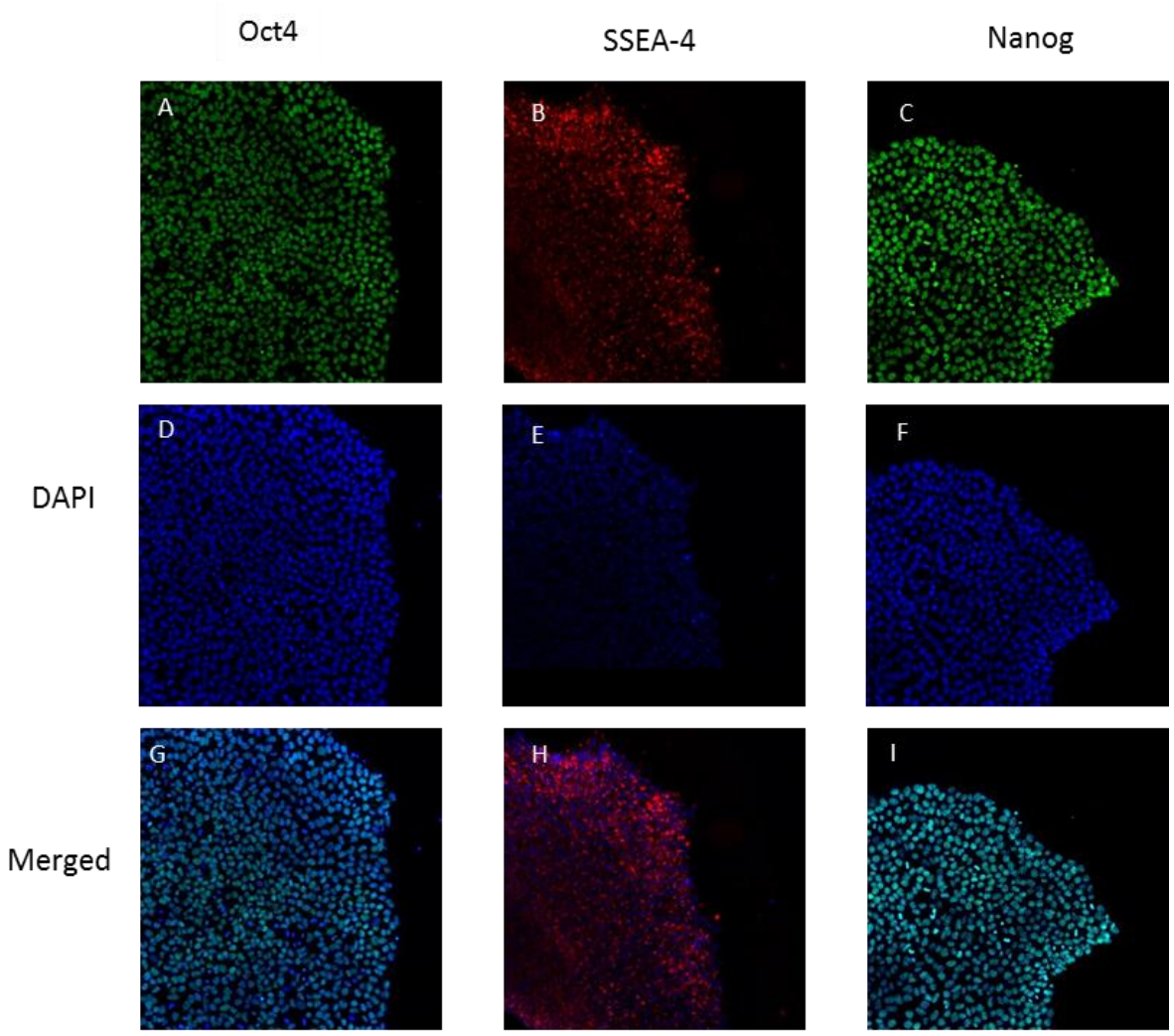


Figure 16: Immunofluorescence staining of Parent iPSCs. Three pluripotency related proteins are stained including Oct4, SSEA4 and Nanog. DAPI is used to indicate nucleus.

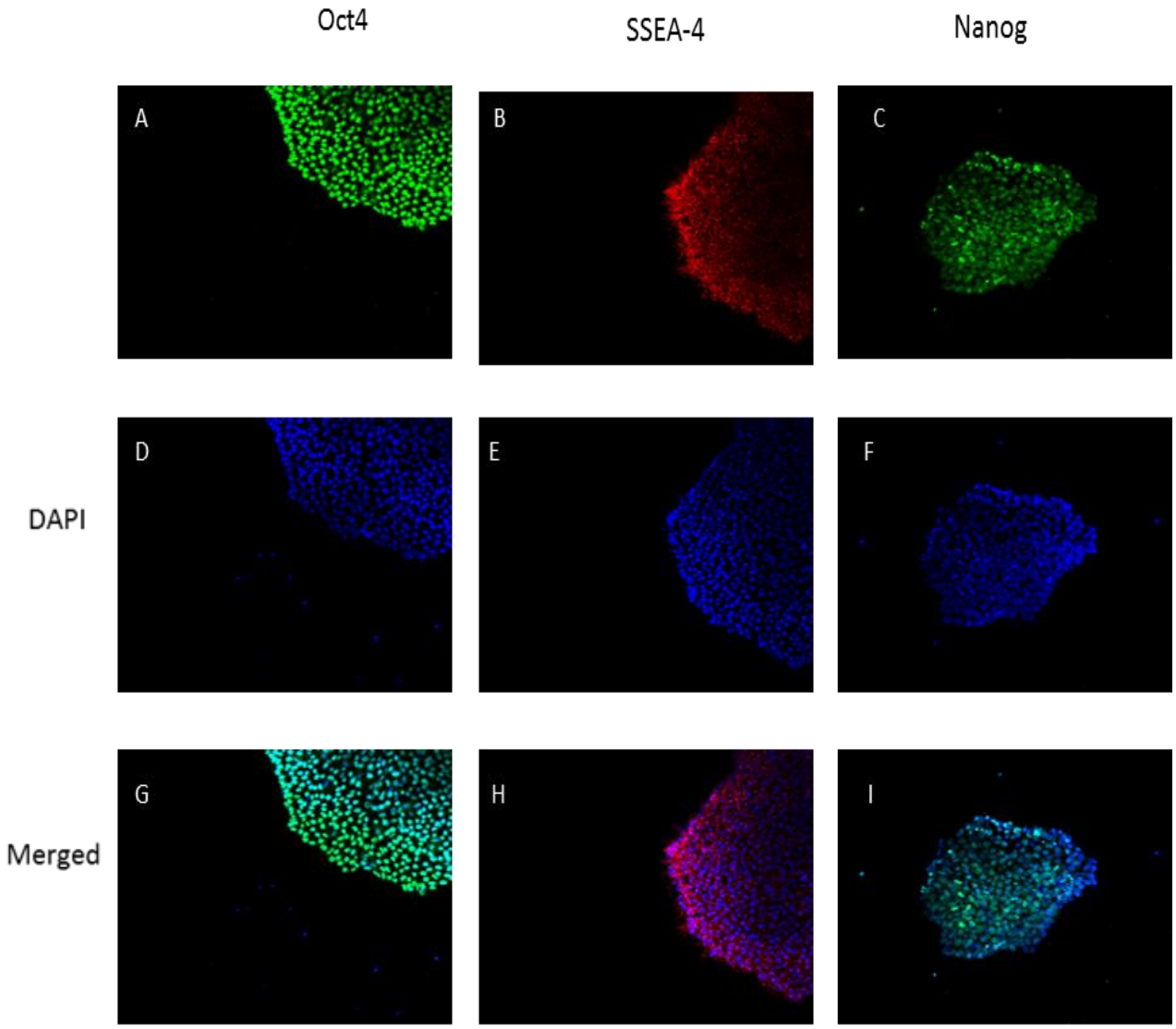


Figure 17: Immunofluorescence staining of PBMC iPSCs. Three pluripotency related proteins are stained including Oct4, SSEA4 and Nanog. DAPI is used to indicate nucleus.

3.5 Generated iPSCs Are Able To Form Teratoma

Morphological and structural analysis of iPSCs are important to indicate pluripotency. However functions of cells are more crucial to demonstrate pluripotency. Therefore, it is vital to show differentiation ability of iPSCs into three germ layer (endoderm, ectoderm and mesoderm) in vivo. To elucidate functional pluripotent ability of iPSCs, I injected iPSC lines

into SCID mice. Since immune rejection is blocked in SCID mice, injected cells could generate differentiated cells.

All injected iPSC lines formed teratoma. Teratoma was stained in a pathology department (American Hospital, Istanbul) and examined by a pathologist (Prof. Dr. Arzu Ruacan). Respiratory epithelium structure is a pattern of endodermal lineage. Pigmented retinal epithelium formation demonstrated the differentiation ability of iPSCs into ectodermal lineage. Teratoma was also differentiated into cartilage which is a mesodermal lineages. Thus, all three germ layer structures were observed including mesoderm, ectoderm and mesoderm **(Figure 18).** This indicates generated cell lines are functionally pluripotent.

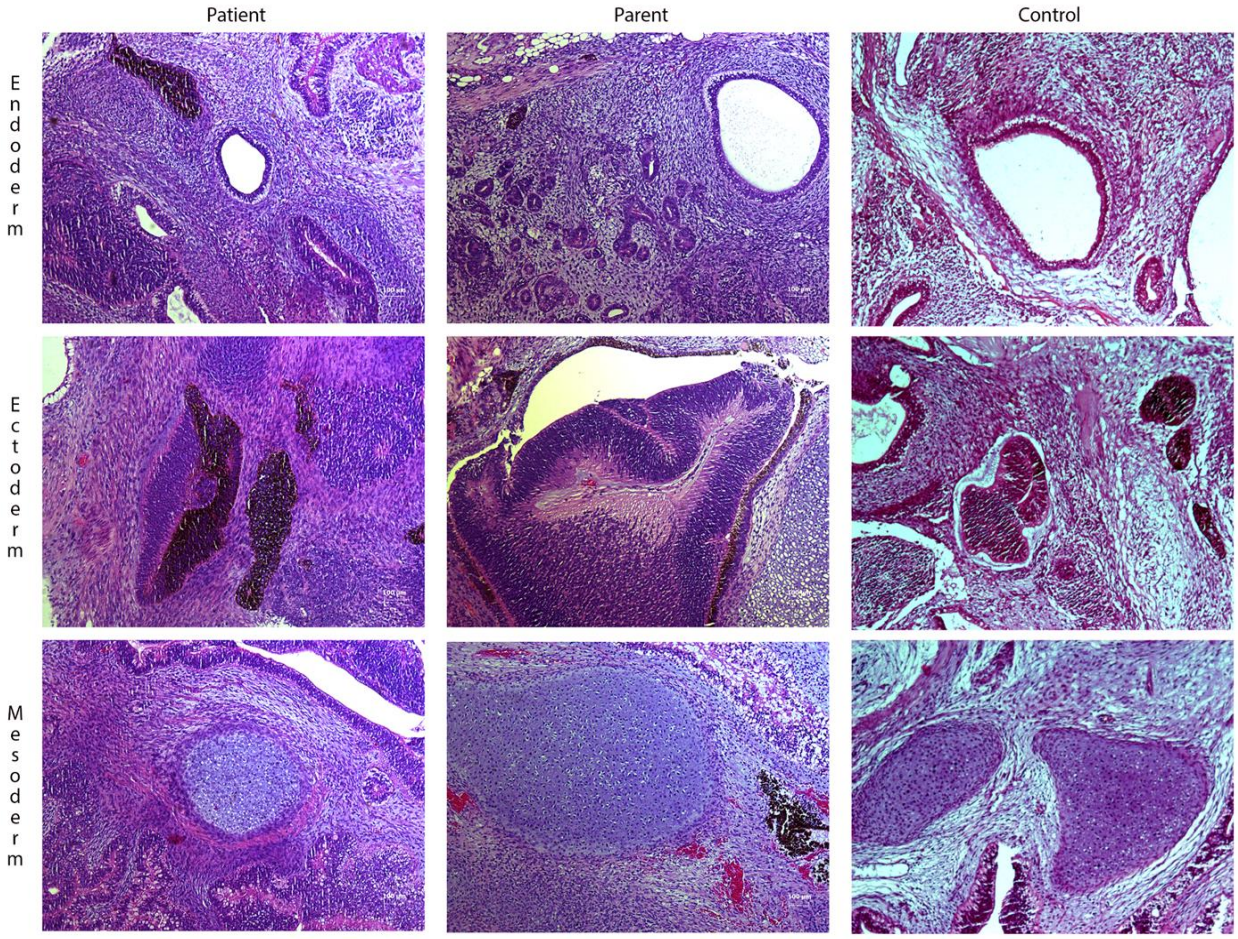


Figure 18: Teratoma formation of injected iPSCs in SCID mice. All three germ layers including endoderm, mesoderm and ectoderm are observed in Teratoma. The results demonstrate the pluripotent abilities of iPSCs. Control group indicates PBMC iPSCs.

3.6 Generated iPSC Lines Are Integration-free

To avoid genomic disruption, I utilized episomal vectors which encode reprogramming factors since episomal vectors do not integrate into genome. However, still there is a low probability for integration. To test for integration possibility, I performed a PCR-based integration assay to genomic DNA. I used primers targeting EBNA sequence which all episomal vectors contain.

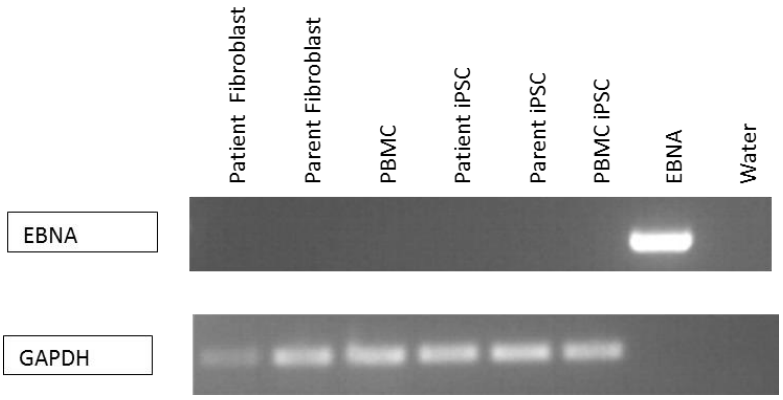


Figure 19: Integration Analysis of iPSCs. PCR-based method was used to analyze integration of episomal reprogramming vectors. EBNA sequence which is encoded by all reprogramming vectors was used to detect integration. Only control sample is positive for EBNA sequence. PCXLE-SK was used for positive control (1 µg).

PCR products were run on an agarose gel. Only positive control which is an episomal vector (pCXLE-SK) was positive for EBNA sequence. All other iPSC lines were negative. Results show episomal vectors are not integrated in the genomic DNA of generated iPSC **(Figure 19)**.

3.7 iPSCs Have Normal Karyotype

To show chromosome abnormalities and number of chromosomes in iPSCs, G-banding was utilized. As it is shown in Figure, all three iPSCs have a normal karyotype for 10 metaphase cell (**Figure 20**, for patient iPSCs; **Figure 21**, for parent iPSCs; **Figure 22**, for PBMC iPSCs).

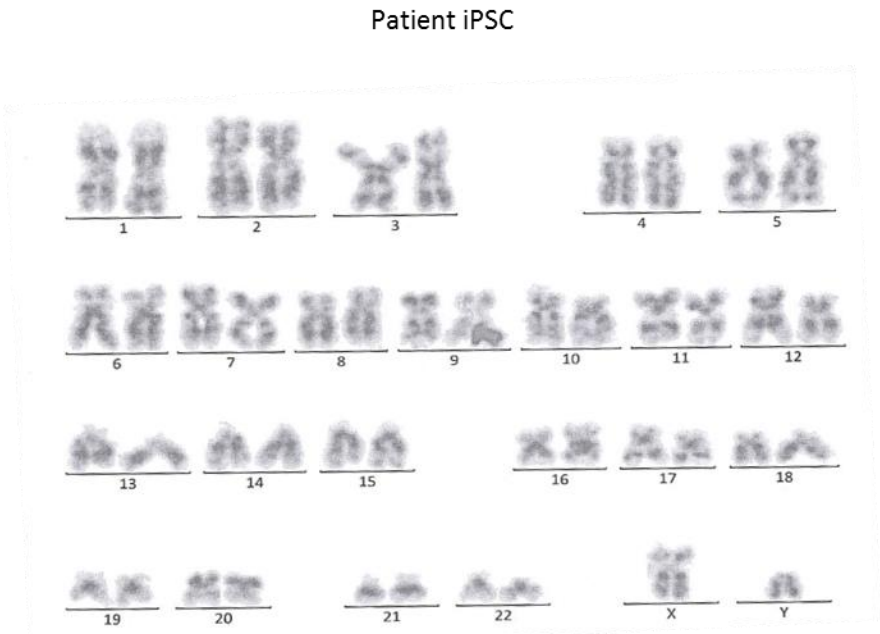


Figure 20: Karyotype analysis of Patient iPSCs. Number of chromosomes is normal with 44+XY

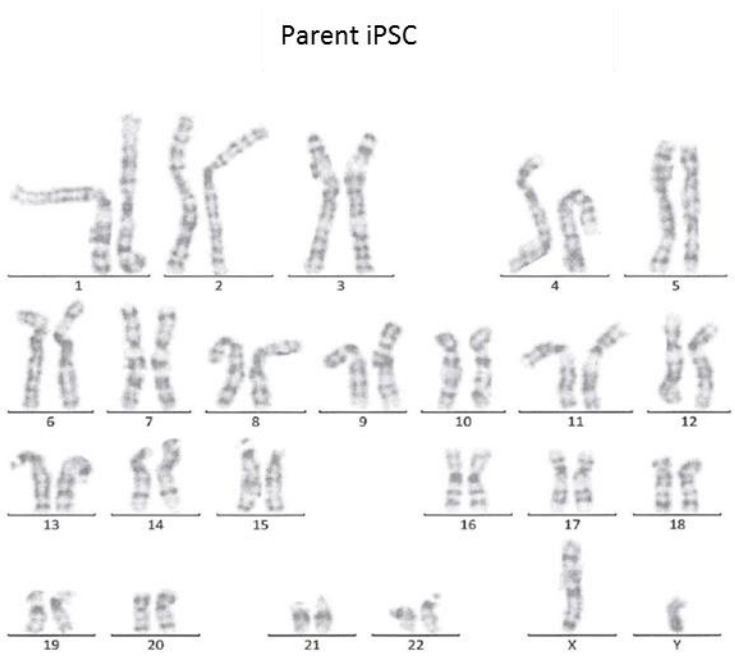


Figure 21: Karyotype analysis of Parent iPSCs. Number of chromosomes is normal with 44+XY

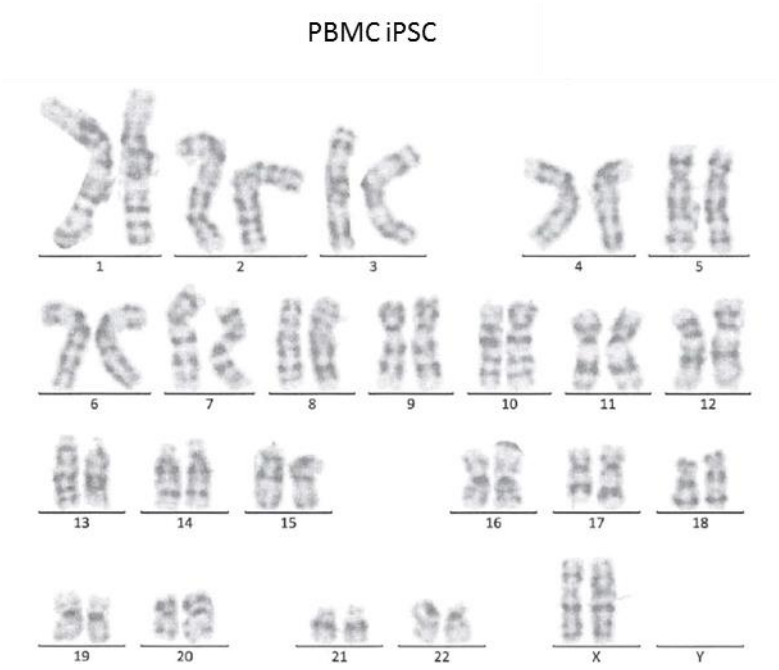


Figure 22: Karyotype analysis of PBMC iPSCs. Number of chromosomes is normal with 44+XX

3.8 iPSCs Were Differentiated Into Macrophages

3.8.1 Embryoid Body Formation Method

To differentiate iPSCs into macrophages, embryoid body formation protocol was used. During protocol, EB morphology was changed **(Figure 23)**. Since macrophages were not produced, modified experiment sets were used **(Figure 24)**.

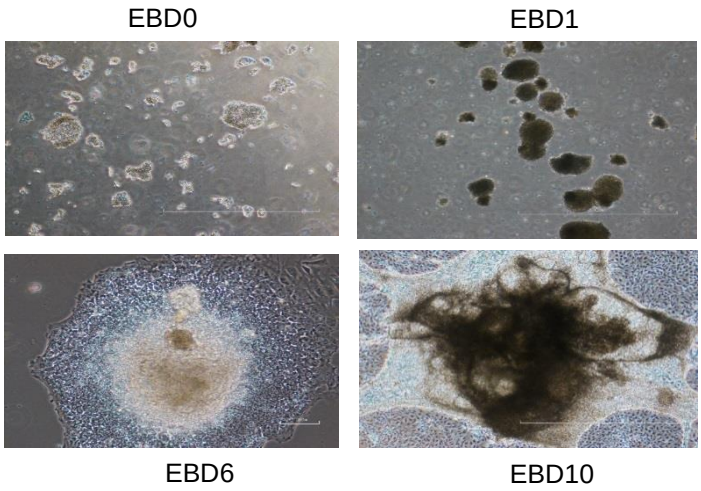


Figure 23: Morphology of Embryoid bodies during macrophage differentiation. On Day 0 and Day 1, EBs are in suspension culture. On Day 6 and Day 10, EBs are attached.

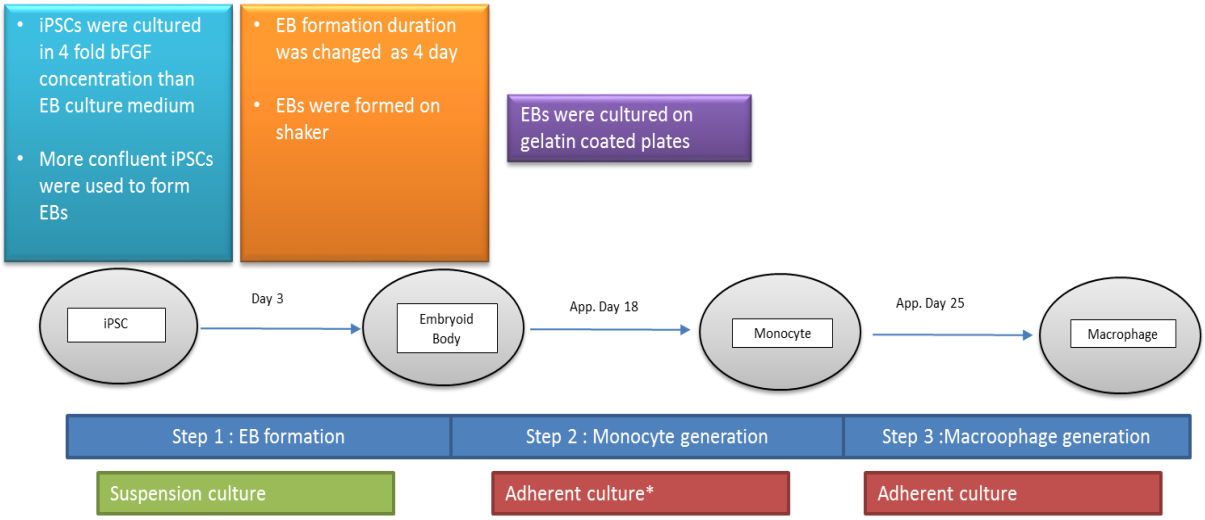


Figure 24: Modifications in macrophage generation protocol from iPSCs via EB formation. Modified steps that are used in the thesis is indicated. Asterics show that EBs are cultured as adherent culture. However, monocytes are released from EBs and suspended in the medium Adapted from reference [73].

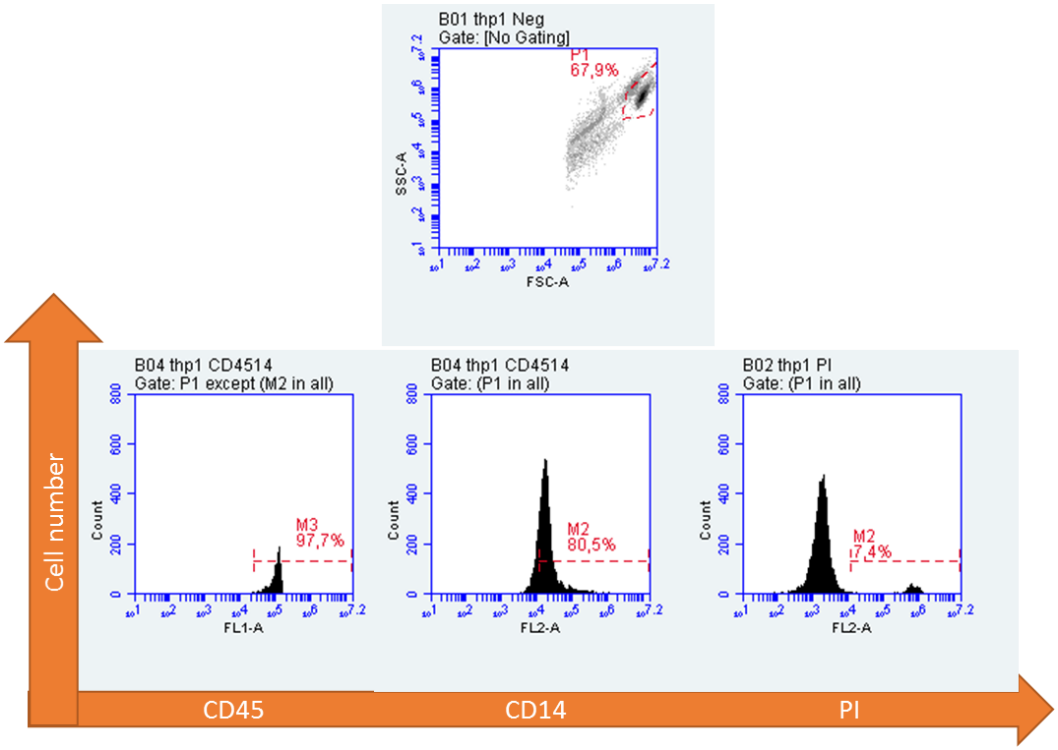


Figure 25: Fluorescent staining of THP-1 cells. As a positive control, THP-1 cells are stained with CD45 and CD14 antibodies.

To evaluate staining protocol, I stained THP-1 cells with CD45 and CD14 antibodies **(Figure 25)**. To show the viability of cells I also stained with PI. PI positive cells indicate dead cells due to lack of dye efflux. Both CD45 and CD14 were expressed on THP1 cells which shows that our staining protocol and antibodies are working **(Figure 26)**.

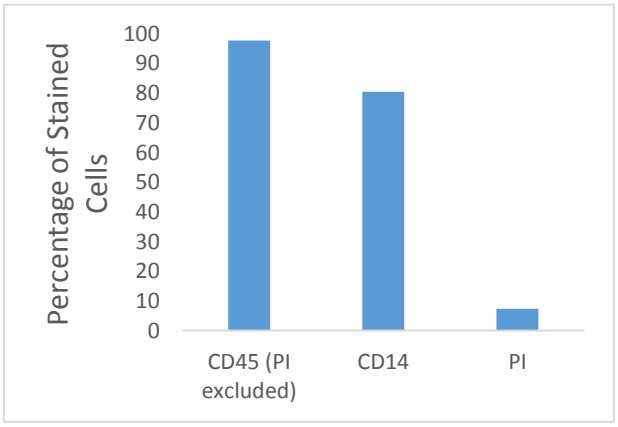


Figure 26: Percentage of stained THP-1 cells with CD45, CD14 antibodies and PI.

First of all, Patient iPSCs were passaged into low-attachment plates to form EBs for 3 days. On day 3, 20-40 EBs were collected and seeded into one well of 6-well plate in EB monocyte differentiation medium. EBs were cultured for 2 weeks and then cells in the medium were analyzed with flow cytometry. Monocytes should be released from EBs into medium. Both CD45 and CD14 positive cells were lower than 1% of cell population. In additionally, 28% of cells were PI positive **(Figure27)**.

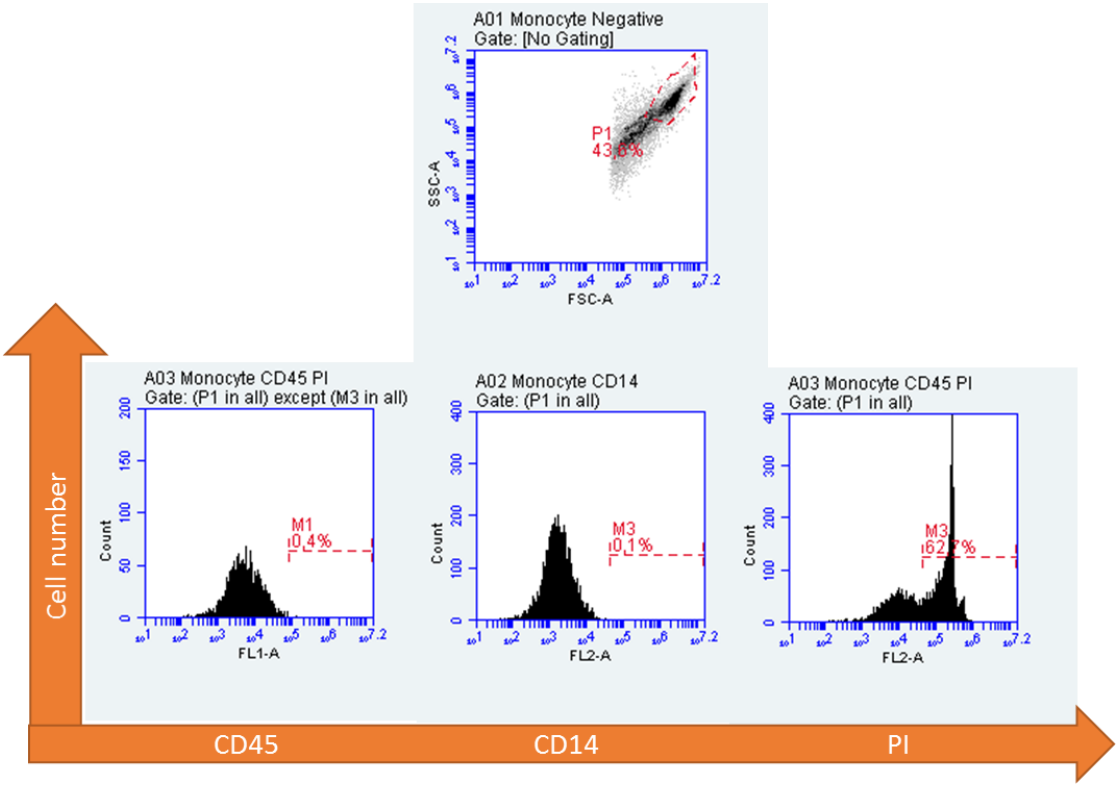


Figure 27: Fluorescent staining of Patient iPSCs-derived monocytes (Week 2). Cells were analyzed after 2 weeks of adherent EB culture.

Since EB attachment efficiency was low, in step 2, plates were coated with 0.1% gelatin. Therefore, EBs were cultured in gelatin coated plates at step 2. However, after 2 week of EB culture on gelatin coated plates, monocyte production was not observed. Approximately 0.1% of cells were positive for CD45 and CD14. However, 25% of cells were PI positive **(Figure 28).**

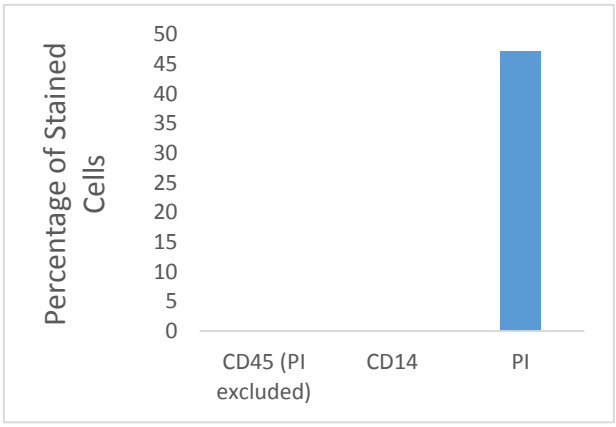


Figure 28: Fluorescent staining of Patient iPSCs-derived monocytes (Week 2). Cells were analyzed after 2 weeks of adherent EB culture.on gelatin.

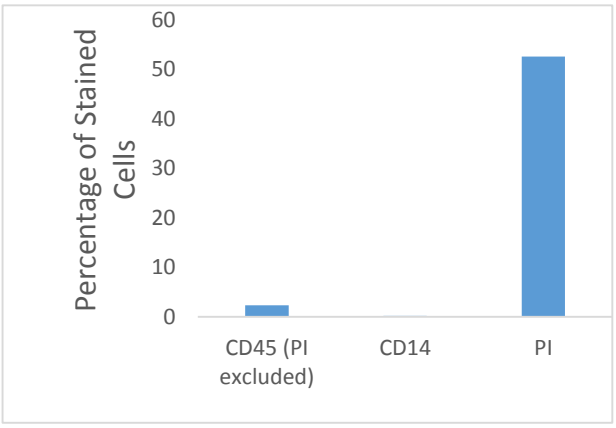


Figure 29: Fluorescent staining of Patient iPSCs-derived monocytes (Week 3). Cells were analyzed after 3 weeks of adherent EB culture on gelatin.

To evaluate the phenotype of EB-released monocytic cells in further weeks of EB formation protocol, cultured EBs on gelatin were cultured for 3 weeks. However, number of positive cells for CD45 or CD14 was not changed **(Figure 29).**

Since the effect of mutation in Patient iPSCs on differentiation ability is not known, cell line was changed with PBMC iPSCs. For this experiment set, PBMC iPSCs were cultured in hESC medium with 4 fold-bFGF concentration. Also, EB formation was done using an orbital shaker. EBs were continuously shaked at 100 rpm for 5 days. Then EBs were cultured on gelatin coated plates in macrophage differentiation medium for 3 weeks. However, monocyte production was not observed **(Figure 30).**

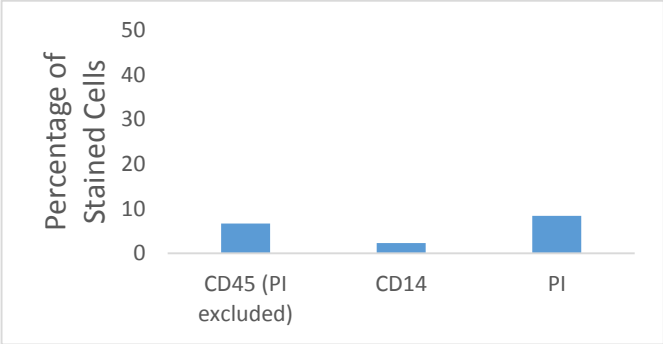


Figure 30: Fluorescent staining of PBMC iPSCs-derived monocytes (Week 3). Cells were analyzed after 3 weeks of adherent EB culture.

As final modification of EB protocol, iPSCs colony numbers were increased to start EB formation. For all previous macrophage differentiation protocols using EB formation, 1 well iPSCs of 6-well plate was transferred into 1 well of 6-well low-attachment plate for EB formation. However, in this final set of experiment, 3:1 passaging ratio was used which means that 3 well of iPSCs were transferred into 1 well of 6-well low-attachment plate. Although the method led the formation of larger EBs, still monocyte production was not observed **(Figure 31) (Figure 32).**

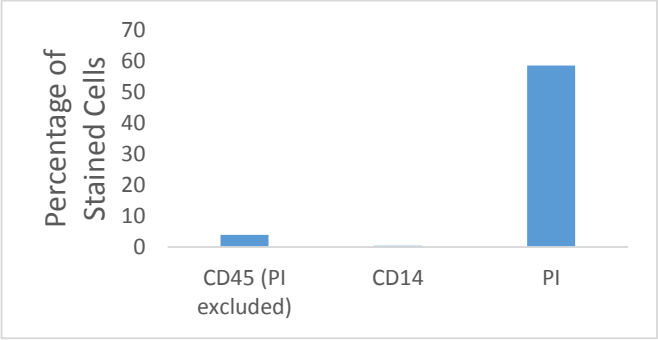


Figure 31: Fluorescent staining of PBMC iPSCs-derived monocytes at 3:1 ratio. iPSCs were passaged at 3:1 ratio to form EB. Cells were analyzed after 3 weeks of adherent EB

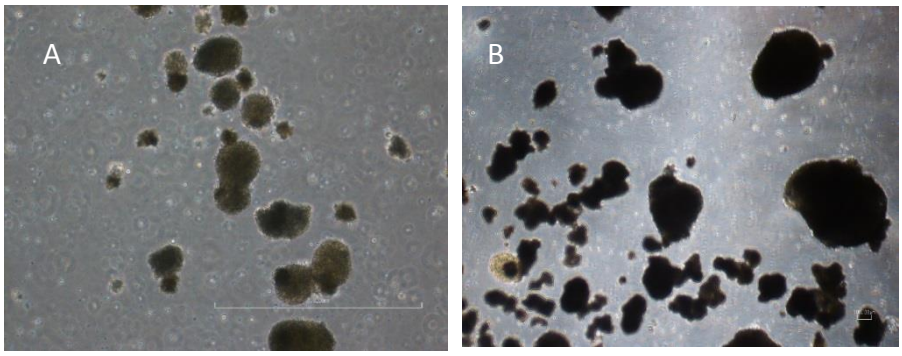


Figure 32: Initial colony number of iPSCs changes EB size. A- 1 well of 6 well plate iPSCs were transfered into 1 well of 6-well low-attachment plate. B- 3 well of 6 well plate iPSCs were transferred into 1 well of 6-well low-attachment plate. Increased number of iPSCs are important to form larger EBs. Larger EBs could produce more monocytes.

3.8.2 Coculture Of iPSCs With OP9

iPSCs were cocultured with OP9 stromal cell line to generate macrophages. OP9 cells were cultured for almost 2 week to reach confluency. iPSCs were cultured on over-confluent OP9 in OP9 differentiation medium for 10 days. On day 10, cells were analyzed by flow cytometer using cell surface markers however monocyte markers were negative **(Figure 33) (Appendix 3a and 3b).**

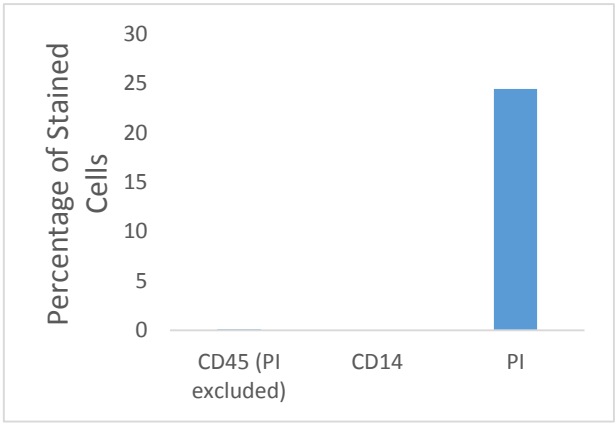


Figure 33: Fluorescent staining of Patient iPSCs-OP9 coculture on day 10.

CHAPTER 4

Discussion

Disease modeling consists of two steps: generation of iPSCs and their differentiation into disease relevant cell types [67], [95]. In this thesis, I generated and characterized an iPSC line which was reprogrammed from fibroblasts of a FMF patient (Homozygous mutant for M694V). For control group, I also generated and characterized iPSC lines from a FMF parent (heterozygote mutant) and a healthy person (wild type). Fibroblasts of patient and parent FMF, and PBMCs of healthy person were reprogrammed using episomal vectors. Thus, I produced integration-free iPSC lines. Generated cells were characterized with various assays to demonstrate that cells are true iPSCs. In the future, generated FMF patient cells can be used for macrophage differentiation which is the disease relevant cell type to model FMF disease.

To generate iPSCs of FMF patient and parent, a skin punch biopsy was obtained from FMF patient and parent. Fibroblast cell lines (thereby will be named FMF patient fibroblasts and FMF parent fibroblasts) were then generated and stocked for further studies. Then, I introduced episomal vectors that encodes pluripotent transcription factors including Oct4, Sox2, Klf-4, L-Myc, Lin28 and also sh-p53 into FMF fibroblasts by utilizing an electroporation system (Neon Transfection System). Electroporation of episomal vectors is an inefficient method to reprogram cells. Therefore, to increase reprogramming efficiency I used episomal vector that encodes sh-p53. After one week following electroporation, I cocultured electroporated FMF fibroblasts with mito-MEFs. Two weeks later of coculture, iPSCs colonies were formed. Even though colonies were formed, several assays were needed to demonstrate pluripotency [65]. Hence, I expanded iPSCs (thereby will be named FMF Patient and Parent iPSCs) for further analysis. After all, I demonstrated that formed colonies are true FMF patient and parent iPSCs by utilizing characterization tests.

I reprogrammed peripheral blood mononuclear cells (PBMCs) of a healthy person to possess a control group. PBMCs were used since isolation is easier than skin biopsy. PBMCs were collected with Ficoll-Plaque according to their density gradient. PBMCs were then expanded in cell culture with proper growth factors. After one week, I transfected episomal reprogramming vectors into PBMCs using Neon transfection system. After transfection, cells were cocultured with mito-MEFs. As in fibroblast reprogramming, after two weeks of

coculture, colonies were formed. Then I expanded and characterized colonies to demonstrate that colonies are true iPSCs.

In order to show that reprogrammed cells are self-renewable and pluripotent several assays were needed to be done. As a first assay, I evaluated the pluripotent gene expressions of reprogrammed cells. Pluripotent gene expressions were compared with a control which is human ESC line, H1. Endogenous Oct4 and Sox2 expression levels of all three iPSCs (Patient, Parent and Healthy) are relatively low than H1-ESCs. (Note that RNA of H1-ESCs were isolated previously in USA by Tamer T. Onder.) On the other hand, Nanog gene expression is higher in the iPSCs. Differences of the gene expressions could be caused by numerous factors including culture conditions, reprogramming method and cell source. For instance, H1-ESCs were cultured on Matrigel but iPSCs were cultured on mito-MEFs. Despite of the slight differences in gene expression levels, generated iPSCs could be considered as true iPSCs due to presence of the pluripotent gene expression. However, expressions of pluripotency genes are not sufficient for reprogramming. For the functionality of expressed genes, transcripts should be translated into protein. Therefore, I examined the pluripotency-related protein levels in the iPSCs.

Protein expression could be analyzed by either western blot or immunofluorescence staining. I aimed to visualize proteins that are related with pluripotency in the iPSCs colonies. Therefore, I fixed and stained iPSCs colonies with fluorescence conjugated antibodies. Main pluripotency markers were aimed to be illustrated at protein level such as Oct4, SSEA-4 and Nanog. Staining of FMF patient, parent and healthy iPSCs colonies showed that pluripotent gene transcripts are translated into proteins. After all, gene expression and immunofluorescence staining assays are not enough to demonstrate pluripotency ability of iPSCs. To asses pluripotency I performed Teratoma formation assay.

Teratoma formation is a powerful method to evaluate the pluripotency. Thus, I injected iPSCs into SCID (severe combined immune deficient) mice intramuscularly. Since mice were immune deficient, they could not reject the injected iPSCs. After 8 or 10 weeks following iPSCs injection, a mass on the leg of mice was formed which is called teratoma. Teratoma is a tissue-like structure that contains three germ cells. I isolated teratoma from mice and fixed them in formalin. Hematoxylin and eosin staining was performed to illustrate germ layer patterns. Formation of germ layer patterns indicates that generated iPSC lines can functionally differentiate into three germ layer cells.

Currently, various iPSCs generation methods are available [96]. Some methods such as lentiviruses lead to a change in the genome of the host since vectors integrate into genomic DNA [87]. In disease modeling, genetic background of the iPSCs should be as similar to patient somatic cells as possible. To avoid the disruption of genomic DNA, I reprogrammed somatic cells using episomal vectors. Episomal vectors are plasmid-like vectors that do not integrate into host genome [51]. Continuous culture of cells lead to the elimination of episomal vectors. However, there is still a chance for integration. I did a PCR-based integration analysis based on EBNA sequences found in all episomal reprogramming vectors. By using EBNA primers, I did a PCR with genomic DNA of iPSCs. PCR samples were run in agarose gel which showed the amplified EBNA sequences. Results demonstrate that only positive control has EBNA sequence, iPSCs are negative for EBNA sequences. This result indicates that İPSCs are integration-free.

Gene expression analysis, immunofluorescence staining and teratoma formation assay show the self-renewal and pluripotent ability of iPSCs. Due to high telomerase activity, iPSCs can be cultured for a long time. However, long term culture conditions can affect the cell features. One important change could be the chromosomal abnormality. To eliminate the possibility chromosomal abnormality in the iPSCs, G banding was performed [91]. Results demonstrate that chromosomes of the generated iPSC lines are normal.

FMF disease affects various cell types including macrophages, fibroblasts and neutrophils [10]. However, disease phenotype can be demonstrated with higher IL-1β secretion from macrophages. Therefore, in this thesis, I aimed to differentiate iPSCs into macrophages. I used three main differentiation protocols including Embryoid body formation, OP9 coculture and infection with PU.1 and C/EBP-α expressing vectors to generate macrophages from iPSCs [68], [73], [74]. To evaluate the differentiation of iPSC into monocytes or macrophages, cells were collected and stained with anti-CD14 and anti-CD45 antibodies at the end of the all differentiation protocols for flow cytometric analysis. While CD14 is a monocyte/macrophage marker, CD45 is a pan-leukocyte marker that is expressed on all leukocytic cells [97], [98].

Embryoid body formation protocol consists of three steps: first step is formation of EBs. At second step EBs release monocytes. At the final step, generated monocytes differentiate into macrophages. It is suggested that monocyte production is starts in the second week of EB culture [73]. Therefore, I collected the medium to analyze cell surface markers of cells. Flow cytometer results indicated that almost less than 1% cells were CD14 or CD45 positive (Figure 24). However, 80% CD14-CD45 positive cells were expected. Since for each Flow

cytometer analysis, 1 x 10^4 cells were counted, yielded monocyte number is very low for further analysis of monocytes. Thus, by using EB formation protocol, monocytes were not produced efficiently.

Although the reason why monocytes were not produced was not clear, one possibility could be the numbers of EBs attached in second step. To increase attachment of EBs to the plate surface, plates were coated with gelatin [99]. Even though attached EB numbers were increased in the coated plates, monocyte differentiation was not observed (Figure 25).

Since monocyte differentiation was not observed in second week of EB culture, I continued the culture for one more week which monocyte production should be increased [73]. Hence, medium of 3 week cultured EBs were collected and analyzed. Although 8% of cells were CD45 positive, 60% cells were also stained with PI (Propidium iodide) (Figure 26). Since PI staining is an indicative of dead cells, most probably CD45 positive cells are false positive which means cells were dead. This results showed that 3 week culture of EBs do not produce monocytes.

Since, the role of pyrin on macrophage differentiation is not known, there could be a chance that pyrin mutant iPSCs may impair differentiation into monocytes/macrophages [100]. To eliminate this possibility, I differentiated PBMC iPSCs. In addition, I slightly changed the differentiation protocol. Before starting the protocol, iPSCs were cultured in hESC medium with 4-fold higher bFGF concentration. In addition to bFGF concentration, iPSCs were incubated on shaker for 5 days to form EBs. It is suggested that shaking could generate larger and homogenous EBs which are important for monocyte production. However, none of these modifications resulted in monocyte generation (Figure 27).

To generate larger EBs, I increased the iPSCs colony number at the beginning of EB formation step [86]. Thus, I combined 3 well iPSCs into 1 well of 6-well low-attachment plate. Increased number of iPSCs formed larger EBs (Figure 28). But larger EB formation was not enough to produce monocytes (Figure 29).

Since monocyte production was failed in EB formation protocols, I then cocultured iPSCs with OP9 cells. OP9 cell line is a mouse bone marrow stromal cell line which commits iPSCs towards to hematopoietic lineage [68]. OP9 coculture protocol consists of two step. In first step, iPSCs differentiate into hematopoietic cells via coculture with OP9. Then, in second step, cells harvested and cultured for macrophage differentiation with proper cytokines such as M-CSF or GM-CSF. Thus, iPSCs were cultured on confluent OP9 cell line for 10 days. On

day 10, cells were analyzed with flow cytometry. However, neither CD45 nor CD14 were expressed on cocultured cells iPSCs (Figure 30).

I also enforced macrophage-specific gene expression in İPSCs via viral infection. Since viral infection of iPSCs is inefficient to express exogenous genes, I infected iPSCs which are cocultured on OP9 for 1 day [74]. Thus, PU.1 and C/EBP-α expressing virus infected cocultured iPSCs. Culture conditions were same with OP9 coculture protocol. Cells were collected on D7 and gene expression was analyzed (Figure 32). Both infected and uninfected coculture systems expressed Runx1 which is a hematopoietic stem cell marker. Thus, results showed that cocultured cells are committed toward to hematopoietic lineage. Viral infected iPSCs gave a preliminary data. Still, protocol is needed to be repeated to analyze surface markers of differentiated cells.

One reason for failed monocyte/macrophage production could be epigenetic memory. Cell origin may affect the differentiation ability of iPSCs due to epigenetic memory [101]. Since homozygous and heterozygous mutant iPSCs were generated from fibroblasts, differentiation may not be done properly. In contrasts, PBMCs-derived iPSCs were not differentiated. A method to resolve this issue could be the usage of chemicals that regulates epigenetics of the cells. Thus, iPSCs could produce more monocytes.

Generated iPSCs were expanded and differentiated as heterogeneous colonies. Therefore, clonal variation could affect the differentiation potential [102]. Some clones could be differentiated easily then other clones. Since I have utilized heterogeneous iPSC clones, production of monocytes may be reduced. To eliminate clonal variation, random clones can be collected and expanded in separated wells. By differentiating expanded clones, proper monocyte-producing clones can be selected and used in disease modeling.

Another reason could be genetic variation of iPSCs. Genetic background of individuals may have a role in iPSCs differentiation potential [102]. Even though a protocol indicates that monocytes can be produce from iPSCs, iPSCs with different genetic backgrounds may need optimized protocols. Thus, each iPSCs clones may need specific differentiation protocols.

Differentiation abilities of iPSCs can be decreased with increased passage numbers [73]. I have used iPSCs which passage numbers were between 25 and 40 for differentiation protocols. Usage of iPSCs with lower passage numbers can increase the monocyte production percentage.

Genetic mutations that affects the differentiation ability of iPSCs can be another reason for failed monocyte differentiation. iPSCs may acquire mutations during reprogramming process or culturing for expansion [103]. Also there might be some mutations in the source cells. To eliminate the mutations which could change the differentiation ability of reprogrammed cells, I used integration-free reprogramming method. However, even this method may lead mutations. Therefore, various clones can be selected and differentiated to find out proper clones for differentiation.

The reason that why monocytes and macrophages were not generated is not clear, yet. Primary cell sources, reprogramming methods and culture conditions can affect the differentiation efficiency. Therefore, in the future, various protocols can be utilized to generate macrophages. Differentiation of iPSCs into macrophages will let us to examine IL-1β secretion and caspase-1 activation in iPSCs-derived patient, parent and healthy macrophages. The expectation is to demonstrate that patient macrophages secrete more IL-1β under inflammation conditions than parent and healthy macrophages [5]. Confirmation of higher IL-1β secretion in macrophages upon stimulation will be an indicator that our generated iPSCs can be used in FMF disease modeling. Thus, other cell types that are relevant with FMF disease including neutrophils and fibroblasts can be generated with proper differentiation protocols for further analysis of FMF disease.

As a result, I generated and characterized FMF Patient, Parent and Healthy iPSCs. To model FMF disease, I attendant to differentiate iPSCs into macrophages which are one of the disease-relevant cell type. Three differentiation methods were utilized including embryoid body formation, OP9 coculture and viral infection. However, efficient monocyte generation from iPSCs were not observed. Therefore, in the future, new protocols will be followed to generate high number of monocyte from generated iPSC lines.

Conclusion

Reprogrammed cell lines promise large number of applications including disease modeling and autologous cell transplantation. Many diseases are modeled including neurologic, psychiatric and immunologic diseases using pluripotent and self-renewal abilities of iPSCs. With the development of new genome targeting tools, generated iPSCs can be recovered. Thus, in the future, molecular mechanism of genetic diseases can be researched more efficiently.

In this thesis, I aimed to model FMF disease which is an auto inflammatory disease. Due to a mutation in MEFV gene, macrophages of FMF patients produce higher level of IL-1β during the attacks. To model the disease, I reprogrammed fibroblasts of a FMF patient using episomal vectors. As control cell lines, somatic cells of a FMF parent and a healthy person were reprogrammed. Although episomal vectors do not integrate into host genome, I performed a PCR-based assay to eliminate integration possibility. Results indicated that generated iPSCs were integration-free. To demonstrate that reprogrammed cells are bona fide İPSCs, I have done characterization tests. Gene expression analysis showed that pluripotent genes were expressed. Immunofluorescence staining demonstrated the translation of pluripotency-related proteins in the generated cells. Teratoma assay showed the differentiation ability of iPSCs including mesodermal lineage by forming cartilage cells. Hence, iPSCs can be differentiated into other mesodermal lineage cells which in this case monocytes and macrophages. Hence, I established iPSCs lines from FMF patient, parent and healthy person.

Since macrophages are one of the FMF-related cell types and their stimulation can show the FMF disease phenotype in vitro, I aimed to differentiate iPSCs into macrophages. To produce macrophages, I followed three protocols including embryoid body formation, coculture with OP9 cell line and viral infection. However none of these protocols were yielded monocytes and macrophages. FMF patient iPSCs were firstly used for EB formation protocol. However, monocytes which were supposed to be produced prior to macrophages were not generated. Even though myeloid cell differentiation is not deficient in FMF patients, some studies speculate that Pyrin is important for production of myeloid cells. Therefore, a reason could be that Pyrin has a role on myeloid lineage differentiation which as a result mutated Pyrin could not support the proper differentiation in EB formation protocol. Hence, instead of FMF Patient iPSCs I differentiated Healthy iPSCs into macrophages through EB formation

protocol. However, result was not changed; monocytes were not produced. Failed differentiation of both cell types lead me to change differentiation protocol. I then performed coculture and viral infection protocols. Due to long term culture conditions, I couldn't complete protocols. I only checked the differentiation marks at the end of the first step of protocols. Mesodermal and myeloid lineage markers were positive for cells which are in both coculture and viral infection protocols. However, still protocols are needed to be completed to obtain useful results.

As a result, protocols needed to be optimized to produce macrophages from iPSCs to model FMF disease in the near future. Confirmation of disease phenotype of iPSC-derived macrophages will allow the usage of generated iPSC lines in the other applications such as drug screening or differentiation into other cell types such as neutrophils. Thus, molecular basis of FMF disease can be deeply analyzed. Furthermore, generated mutant iPSCs can be corrected with genetic tools such as CRISPR/Cas9 system to understand contribution of mutations to FMF disease. In addition to studies that aim to understand disease mechanism, new treatment approaches can be tested. For instance, drug screening may be evaluated new chemicals that recover the disease phenotypes. Thus, generated iPSC lines in this thesis, promise a useful source to study FMF disease in the future.

APPENDİX

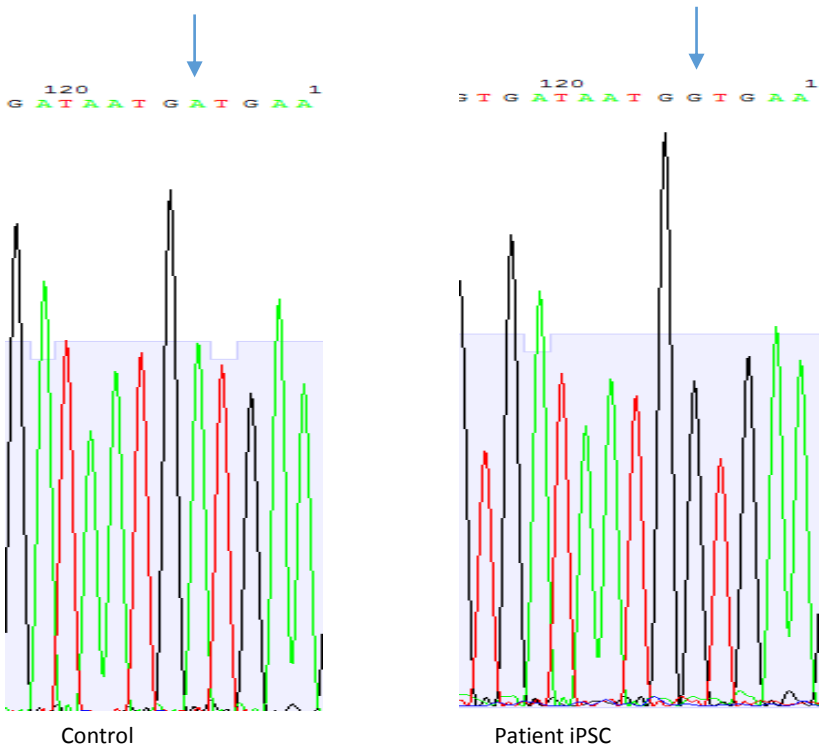
Appendix 1

Licensee:	**Kerem Fidan**
License Date:	December 13, 2015
License Number	3767240368989
Publication	Genes and Immunity
Title	The regulation of MEFV expression and its role in health and familial Mediterranean fever
Type of Use	Reuse in a dissertation/thesis

Appendix 1: Copy Rights of Figure 7.

Appendix 2

Genomic DNA from homozygous mutated iPSCs (Patient iPSCs) were isolated, and exon 10 of MEFV gene was amplified by PCR using the following primers: Fwd: TTCCAGAAGAACTACCCTGT, Rev: TGCATTTCCCATAGCAGCTA. To indicate the presence of homozygous single nucleotide mutation, Sanger sequencing was performed.



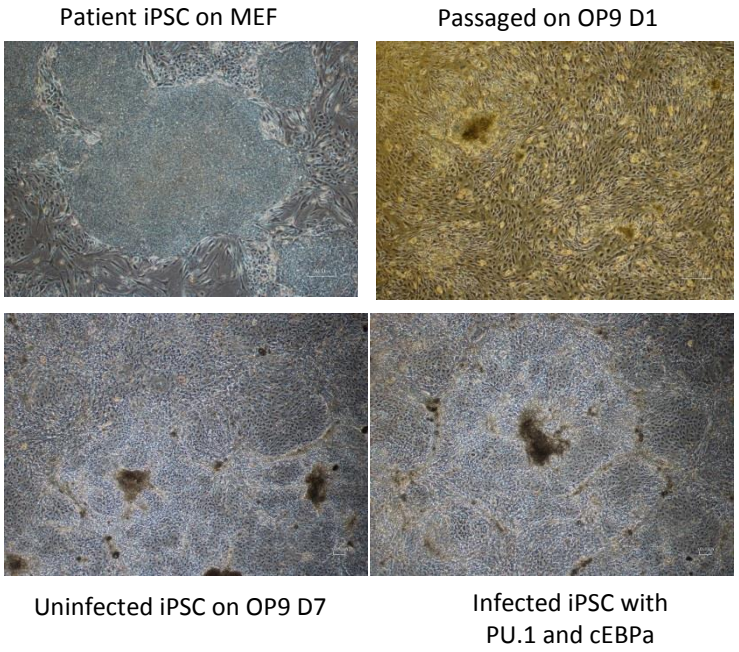
Appendix 2: Mutation detection in patient iPSCs. Mutation in the *MEFV* gene leading to M694V is indicated in iPSCs (Blue arrows point the mutated nucleotide in patient iPSCs)

Appendix 3

Viral Infection Of iPSCs

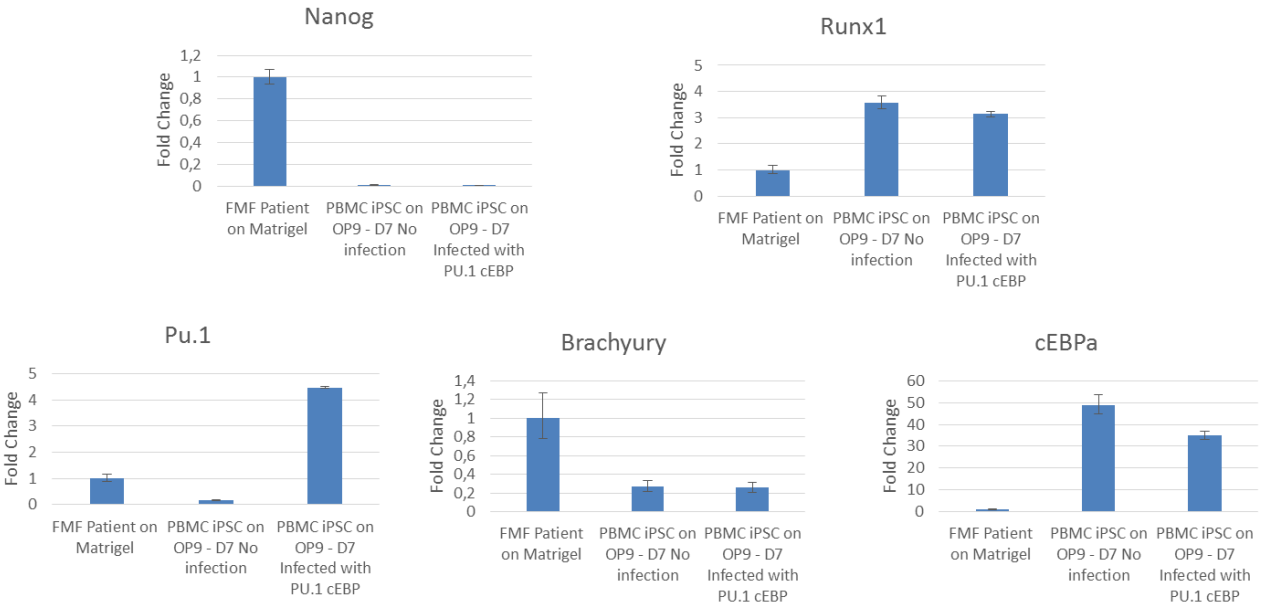
PBMC iPSCs were cultured in normal hESC medium on mito-MEFs to expand. iPSCs were cultured on OP9 as in OP9 co-culture protocol. However, after one day of co-culturing OP9 and iPSCs, cells were infected with PU.1 and c-EBP-α expressing virus. Medium was changed at each 3 days. Cells were collected on day 7 to analyze gene expressions [74].

Since viral infection of undifferentiated iPSCs is inefficient, I infected iPSCs that are cocultured with OP9. Thus, I aimed to enforce expression of main macrophage-specific genes, PU.1 and C/EBP-α, in iPSCs. Morphologies of infected and uninfected cocultures are similar to each other**.**



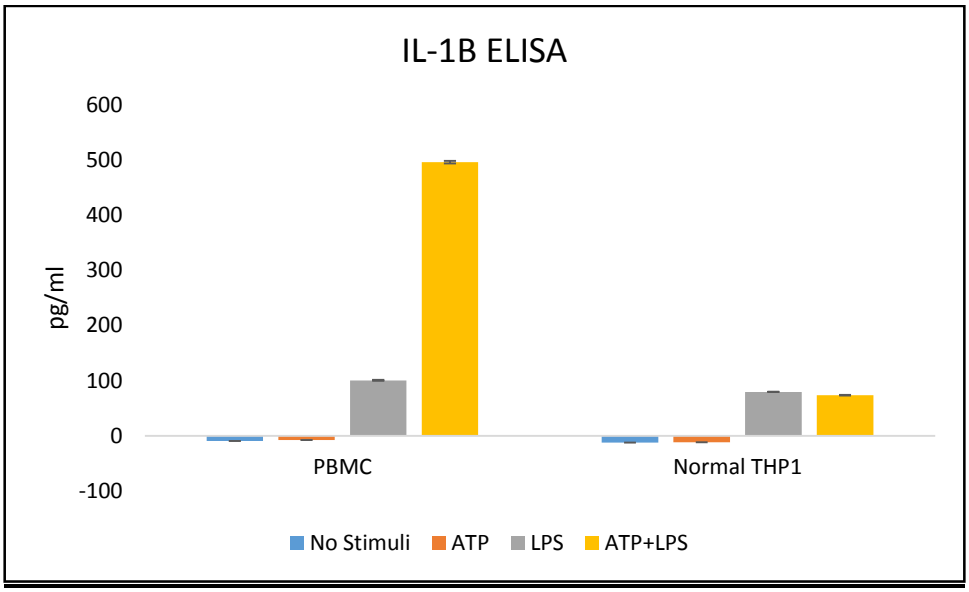
Appendix 3a: Morphology of Patient iPSCs and OP9 coculture. Infection of iPSCs-OP9 coculture with PU.1 and C/EBPα expressing vectors changed morphology slightly different than uninfected coculture system.

Gene expression of infected and uninfected iPSC-OP9 cocultures were analyzed. Nanog was not expressed in cocultured iPSCs. Runx1 was expressed in cocultured iPSCs. However, Brachyury was expressed at lower level. PU.1 was expressed only in infected iPSCs but not uninfected one, however both cocultured iPSCs expressed cEBP-α.



Appendix 3b: Gene expression analysis of cocultured iPSCs. Cocultured iPSCs with OP9 were infected with PU.1 and C/EBP-α expressing vectors. Vector infected iPSCs Express slightly more Runx1 which is an early hematopoietic stem cell marker. Cocultured iPSCs differentiated and do not express Nanog. Infection significantly increased Pu.1 expression. Thus, specific gene expressions can be enforced by viral infection. However, to differentiate cells to monocytic lineage more experiments are needed to be done.

Appendix 4



Appendix 4: IL-1β ELISA results of LPS/ATP treated PBMC and THP1. PBMCs and THP-1 cells were treated with 1 ug/ml LPS for 4 hours and 1 mM ATP for 15 minutes. Supernatant was used for Elisa test. PBMCs are isolated from a healthy person. THP-1 cells are monocytic cell line. Both cell lines were cultured in RPMI medium with 10% FBS. Both PBMC and THP-1 cells secreted IL-1β in the presence of LPS. However, ATP stimulation increased the IL-1β secretion in the PBMCs but not THP-1. In the future, the LPS/ATP stimulation and IL-1β Elisa test can be used for iPSCs-derived macrophages to evaluate disease phenotype.

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