Research Article



Highly efficient ESC genome editing with CRISPR/Cas9 for production of laboratory models

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Abstract

Background: Beta-thalassemia is a group of hereditary blood disorders caused by mutations in the β-globin gene cluster resulting in variable phenotypes ranging from severe anemia to clinically asymptomatic individuals. This study aimed to produce an in vitro model of β -thalassemia using CRISPR/Cas9 as an easily programmable, fast, more powerful, and efficient technique.

Materials and Methods: Guide RNA (gRNA)-Cas9 co-expression vectors were used for embryonic stem (ES) cell nucleofection. PCR, T7EI, and Hbb-b1 gene sequencing tests were done on extracted DNA to evaluate gene mutation. Following erythroid differentiation of ES cells, analysis of hemoglobin genes and erythroid transcription factors were assessed using a quantitative reverse transcription-polymerase chain reaction.

Results: Sequencing data associated with clone 31 confirmed the deletion of 851 nucleotides between exon 2 and 3 in an Hbb-b1 allele in this clone and Indel mutation in exon 2 (-40bp/+38bp) from another allele of Hbb-b1. Significant expression of erythroid transcription factors was observed in wild type, Hbb-b1-/- and Hbb-b1-/- groups. The hbb-b1 gene expression in the Hbb-b1+/- group significantly decreased, although the Hbb-b1-/- group had zero expression.

Conclusion: Utilizing an efficient erythroid differentiation method on the CRISPR/Cas9-mediated Hbb-b1 knock-out in ES cells provides accessibility to the laboratory thalassemia model. This method could be used to produce a mouse model of β-thalassemia intermedia (Hbbth1/th1 mice), which are required for the identification of the molecular basis of β -thalassemia and enable testing of the therapeutic approaches such as the recovery of functional β or γ hemoglobin chain.

Keywords: Beta, thalassemia, CRISPR-Cas9 system, Hbb-b1, Mouse embryonic stem cell

1 Introduction

Breast One of the common monogenic diseases is the hereditary disease of hemoglobin. Thalassemias are a group of genetic diseases of hemoglobin synthesis caused by the reduction of production or lack of production of one or more globin chains and are divided into different categories depending on this. Beta thalassemia is an autosomal recessive inheritance that affects a large population worldwide yearly. In order to understand the importance of thalassemia, it should be noted that about 4.4% of every 10,000 live births have thalassemia (1). There are about 2 million thalassemia carriers in Iran, and the distribution of its prevalence is concentrated in the north and south of Iran (2).

The primary complications of beta thalassemia, which lead to the imbalance of α/β -globin synthesis during a person's life, are caused by genetic defects. Common treatments include blood transfusion and iron chelators to reduce iron load. Allogeneic bone marrow transplantation is the only potential treatment option used in a limited way due to the limited availability of BM donors with appropriate HLA matching with the patient and the possibility of GVHD.

The thalassemia treatment methods used so far have not only had high social and economic costs but have also been Different types of mouse models with thalassemia that have been utilized so far are mentioned in a review study in 2015; for example, exposure to ethylnitrosourea can caused removing some fragments of the regulatory region in β -globin gene to create a mouse model of beta-thalassemia. Further investigation showed that in the case of the initial presence of the mutation in the mouse, this exposure could increase the occurrence and cause anemia(3).

Aoli Yang and colleagues designed and presented another β 0 thalassemia mouse model using the "Plug and Socket" system. In this model, a 20 kb fragment containing two genes, β min, and β maj, of adult rats was deleted (4).

Recently, CRISPR technology has been mentioned as an effective tool for correcting genetic abnormalities. Cas9 (CRISPR-associated protein 9nuclease) is used to recognize target genomic DNA and utilizes RNA-DNA base pairing to spot target genomic DNA.

This mechanism can be used for genomic engineering of mammals, such as gene knockout, creating epigenetic changes, checking the number of repetitions of short sequences in a row, and adding a repressor or enhancer to specific positions on used genomic DNA. In the CRISPR/Cas9 system, in order to delete the gene, using guide RNA, the specific position of the target gene is identified and cut.

The laboratory cells that we produced by knocking out the HBB1 gene by CRISPR have the potential to produce a beta-thalassemia mouse model, and this model can be achieved by injecting the produced cells into pseudo-pregnant mice. (Figure 1).

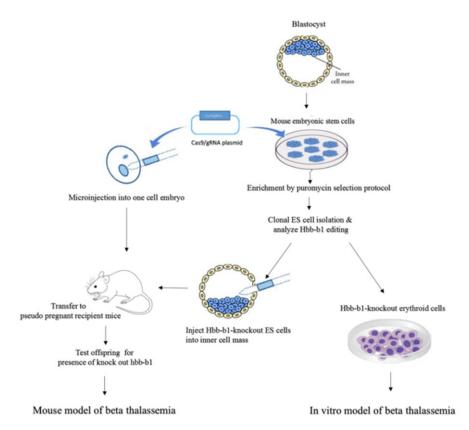


Figure 1. Schematic image for the production of mouse and laboratory models of beta thalassemia using the CRISPR/Cas9 system.

This tool can play a role in the new therapeutic goals of beta-thalassemia and improve the investigation of complications and pathogenesis.

Considering beta-thalassemia's complex clinical and molecular mechanisms, animal models are necessary to understand its pathophysiological mechanisms and hemoglobinopathy and confirm future in vivo therapeutic strategies. Our study will help to treat beta-thalassemia patients.

2.Materials and methods

2.1 Used vectors

Among the two vectors, PX 330 and PX459, available to us, the PX459 vector was chosen. The presence of puromycin as a selective marker in animal cells is its distinguishing feature compared to the pX330 vector. The ability of optimal cultivation and reproduction of this vector is in the Stbl3/4 strain and the bacterial culture medium with ampicillin as a bacterial resistance factor and a temperature of 37°C.

2.2 Bioinformatics studies and gRNA design and plasmid extraction

Using meta tools and online gRNA design sites (E-CRISPR, Deskgen, and DNA 2.0 CRISPR gRNA), gRNAs were observed and sorted based on their target points in protein-coding or non-coding regions, and finally, four guide RNAs were designed. Every type of Complementary sgRNAs was annealed, phosphorylated, and inserted into the digested pX459 with BbsI (Thermo Fisher Scientific). The sgRNA cloning sites were located downstream of the U6 promoter.

A colony polymerase chain reaction (PCR) was run to confirm successful insertion on a peqSTAR 96X Thermal Cycler system (Peqlab, Erlangen, Germany). Removed Plasmids from the positive clones were extracted using a Quick Plasmid Miniprep Kit (Qiagen). At last, sequencing of the plasmid using U6 F primer confirmed the successful cloning.

2.3 Cell culture and transfection

In this study, ES-E14TG2A embryonic stem cells were employed. Because DNA contamination is avoided due to feeder independent growing of these cellsES-E14TG2a cells were cultured in Dulbecco'sDulbecco's modified Eagle'sEagle's medium/nutrient mixture F12 (DMEM/F12) (Gibco) supplemented with 15% fetal bovine serum (FBS) (Gibco), 2 mM sodium pyruvate (Sigma), 1% non-essential amino acids (Gibco), 1% GlutaMAX (Gibco), 103 U/mL Leukemia Inhibitory Factor (ORF Genetics, Iceland), 50 mg/mL penicillin/streptomycin (Gibco), 0.1 mM β-mercaptoethanol (Merck, Germany). Cells were maintained at pH 7.4, 37°C in a water-saturated atmosphere containing 5% CO2.

Before starting a transfection experiment, an effective Amaxa Nucleofector software for ES cells was obtained. Puromycin's Puromycin's lowest dose necessary to cause cell death was also determined.

This research used the Mouse ES cell Nucleofector Kit (Lonza Cat. No. VPH 1001), Amaxa Nucleofector, and the pre-optimized program to transfect the cells.

Briefly stated, 100 L of nucleofector was used to dissolve 2 to 4×106 single cells, and nucleofection was then carried out by combining the cell suspension with 5 g of the CRISPR plasmid. The empty GFP vector (8.5 kb) was nucleofected into ES cells using the four suggested programs (A13, A23, A24, or A30). Flow cytometry (Attune NxT Flow Cytometer, Applied Biosystem) and direct fluorescence microscopy (Nikon TE 2000, Japan) visualization of GFP-expressing cells may then evaluate the transfection effectiveness. Transfected cells were chosen 48 hours after transfection by administering the right amount of puromycin. This way, the probability of selecting successful clones for receiving the plasmid increased. Harvesting and transferring single clones to 96-well plates was done using the method of preparing serial dilutions with the estimation of having a single clone in each well of the 96-well plate. The cells of each well from the 96-well plate were transferred to two wells from the 48 and 96-well plates. The cells of each of the wells of the 48-well plate were subjected to the DNA extraction process and were examined to find clones that had gene deletions. The 96-well plate was also used to have a backup.

2.4 Genome editing

Forty-eight hours after the transfer of plasmids containing gRNA-Cas9 to ESCs, genomic DNA was extracted. The target regions were amplified by PCR method and using a pair of primers designed on both sides of the gRNA binding site, and the target regions were amplified, and the ability of gRNAs to function was evaluated with T7E1 endonuclease.

We performed the T7E1 cleavage assay as instructed by Shen et al. T7E1 (NEB, USA) was applied to hybridized PCR results for 20 to 30 minutes at 37°C and successful hybridization was analyzed using 2% agarose gel electrophoresis. Amplicons were subcloned into a pTG19T vector and transformed into freeze-competent Escherichia coli to verify indel mutations and deletions. Single colonies were sequenced using the M13 F primer to reveal the clonal genotype.

We used two gRNAs simultaneously; in brief, regarding the individual function of each gRNA, due to the addition or subtraction of a small number of nucleotides, it is not possible to identify the desired clone by choosing the primers around the desired region and transferring the product on the electrophoresis gel.

The advantage of using two gRNAs at the same time and checking their function on the ESC genome was the more straightforward observation and monitoring of clones with the simultaneous function of both gRNAs, and as a result, the chance of interrupting and deleting our sequences between gRNAs, and at the same time, it was possible to check the function of a single Each of the gRNAs were also present

2.5 Molecular evaluation

In order to check the simultaneous function of two gRNAs, two primers, F and R, were designed far from the binding region of both gRNAs, and the PCR reaction was performed on both test and control samples (regular clones). Observing a PCR band shorter than the control sample indicates the removal of the target fragment and successful knockout.

3 Results

3.1 Colony PCR result to confirm the presence of gRNA in the vector

Colony PCR was performed with specific primers, and the presence of gRNA in the pX459 vector was confirmed (Figure 2). We used both pairs of primers to ensure the detection of the clone containing gRNA. The presence of the representative band of positive clones indicates the presence and successful reception of the gRNA sequence in the desired clone. (Figure 3).

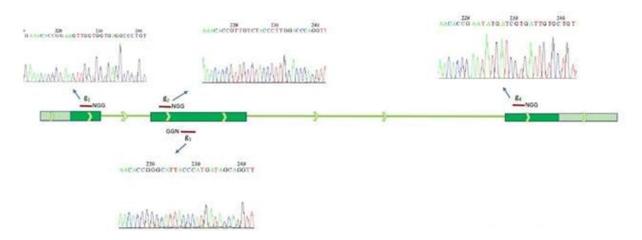


Figure 2. gRNAs sequencing results

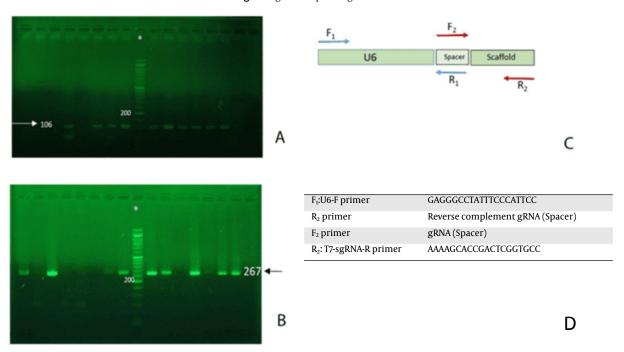


Figure 3. Colony PCR electrophoresis on 2% agarose gel with 100 V voltage. Well* marker with a molecular weight of 50bp, (A) PCR results of the first primer pair (primer F on the U6 promoter and primer R of the complementary and reverse sequence of gRNA), (B) PCR results using the second primer pair (primer F of the gRNA sequence and primer R on the structural region of gRNA). C, Cloning PCR were don using two sets of primers to select successful insertion. D, Primer sequences for PCR amplification, one set of primers (F1/R1) make 267 bp fragment, whereas a second set amplifies a 106 bp fragment (F2/R2). gRNA, guide RNA; PCR, polymerase chain reaction; sgRNA, synthetic single guide RNA.

3.2 Evaluating the results of entering plasmids into ESC

In this study, to introduce plasmids into ESCs, different commercial transfection reagents were used to evaluate the percentage

of entering GFP vectors. All the tests were re-evaluated with repetition and necessary changes in terms of plasmid concentration, availability time of adhesion, and suspension of cells. The best entry percentage for plasmids with an approximate length of 8 kb (pCDH vector) in ESCs was less than 5%, while the percentage of transfer of plasmids with a length of 4 kb (max GFP) to ESCs with optimized methods was estimated to be more than 50%. (Figure 4).

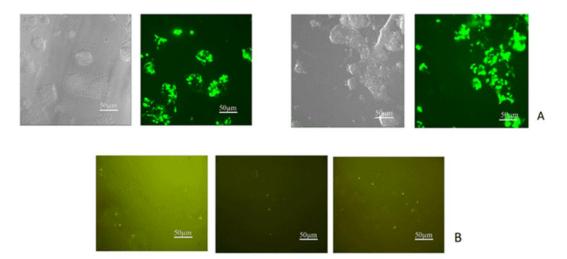


Figure 4. ESCs transfected with GFP reporter vector. (A) Fluorescence microscopy image related to the introduction of kb4 length plasmids (maxGFP), (B) Fluorescence microscopy image related to the introduction of kb8 length plasmids (pCDH vector). Original magnification, x10. Scale bar 50 µm.

Finally, electroporation using the Amaxa-mouse ESC nucleofector kit and selecting the A30 program resulted in the best insertion efficiency (31.5%) for plasmids with an approximate length of 8 kb (pCDH vector), which the fluorescent microscope image and flow cytometer data in Figure 5 follows.

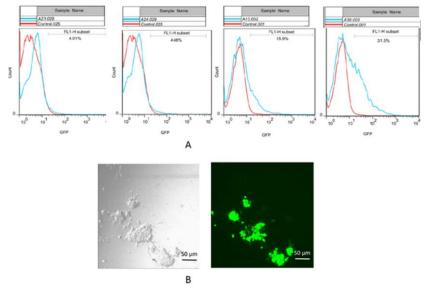


Figure 5. Nucleofected ESCs with GFP reporter vector. (A) Flow cytometry data shows that 31.5% of ESCs are GFP+ using the A30 program (B) Fluorescence microscope image. Original magnification, x10. Scale bar 50 μm.

3.3 Performing PCR, T7EI and Hbb-b1 gene sequencing tests after entering the Cas9/sgRNA plasmid

About 70 single clones were selected and individually transferred to each well of 96-well plates following puromycin selection on g3/g4 nucleofected ESCs. DNAs were collected from 8-10 separate colonies and used for molecular testing. To determine which colonies had successfully removed oligonucleotides, the F1/R1 primer was utilized to assay the simultaneous functions of both gRNAs 3 and 4.

Similar to the outcome of the control group, the detection of a single band at the length of bp1691 indicated that no oligonucleotides had been removed. The absence of the bp1691 band on the electrophoresis gel explained that both alleles had lost the oligonucleotide. In this experiment, except group D, which showed two bands (1691bp and 850bp), all groups showed only one band (bp1691) (Figure 6, A). Reducing the length of the second band (about bp800) showed that at least one clone from this group has the required oligonucleotide.

As a result, the same PCR was carried out on DNA isolated from each of the group's 8 single clones. The PCR result of clone 31 showed two bands with different lengths and the same color intensity (1691bp and 850bp), indicating the knockout of a Hbb-b1 allele (Hbb-b1 +/-). The band, measuring approximately 850 bp in length, was visible and purified from agarose gel before the sequencing test (Figure 6, B).

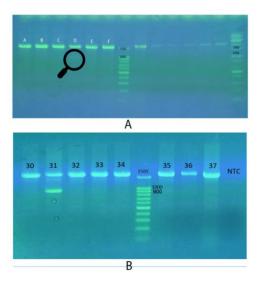


Figure 6. PCR results using F1 / R1 primer pair to detect oligonucleotide deletion in the hbb-b1 gene sequence, well *100bp molecular weight marker and ** well 1kb molecular weight marker.

As validated by sequencing data, the Hbb-b1 allele in clone 31 has an 851-nucleotide deletion between exons 2 and 3 (Figure 7). Clone 31 was then re-nucleofected with gRNAs 3 and 4, and the DNA from each resulting subclone was extracted following puromycin exposure and selection, and the single clones were grown in 96-well plates. A PCR test employs F/R primers designed to examine the function of the two gRNAs separately and together. At last, T7E1 tests were used to confirm the knockout of the Hbb-b1 gene.

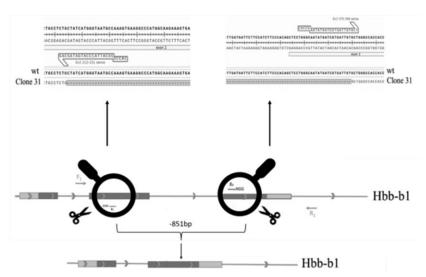


Figure 7. PCR results using F1 / R1 primer pair to detect oligonucleotide deletion in the hbb-b1 gene sequence, well *100bp molecular weight marker and ** well 1kb molecular weight marker.

As shown in Figure 8, clone a has an oligonucleotide deletion in one allele and an indel mutation in the other allele of the Hbb-bt gene.

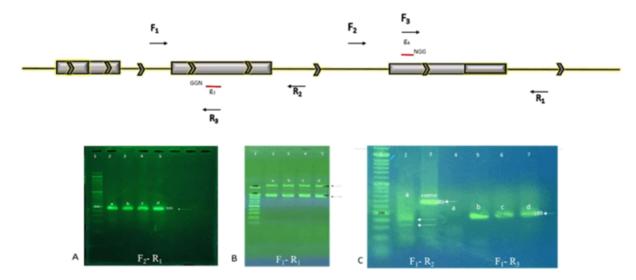


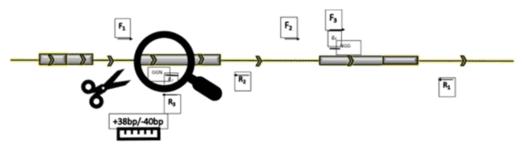
Figure 8. PCR and T7EI results to identify Hbb-b1-/- on the genomic DNA of each of the subclones.

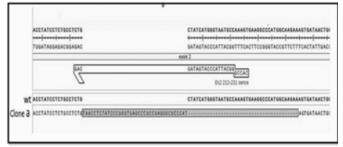
Sequencing data related to clone A confirmed the knockout of the Hbb-b1 gene in both alleles (Figure 9).

Oligonucleotide deletion (-851bp) between exon 2 and 3 in one allele

Indel mutation in exon 2 (-40bp/+38bp) from another allele of Hbb-b1

(A) PCR results using primer pair F3/R1 used to evaluate g4 function. (B) PCR results using F1 / R1 primer pair to evaluate oligonucleotide removal (C) PCR and T7EI results using F1 / R2 and F1 / R3 primer pair to evaluate g3 performance, column 4 (clone A) no observation The target bp 169 bands indicated the successful operation of g3. This result was confirmed by running the T7E1 test (column 2). The Well 1 in Figures A and C is a 50 bp molecular weight marker, and the well 1 in Figure B is a 100 bp molecular weight marker.





Primer sequence	Product size	Application
F1: GTTGGGTGCTTGGAGACAGA	1691	Analysis of cas9/g3 & g4
R1:CAATTAACCATTGTTCACAGGCA		cleavage activity
F1: GTTGGGTGCTTGGAGACAGA	163	Analysis of cas9 /g3 cleavage
R3:CACCGGGCATTACCCATGATAGCAG		activity
F2: GTTGGGTGCTTGGAGACAGA	285	Cleavage activity of cas9 /g3
R2: CCACATGCAGCTTGTCACAG		with T7E1
F2: CACCGAATATGATCGTGATTGTGCT	165	Analysis of cas9/ g4 cleavage
R1:CAATTAACCATTGTTCACAGGCA		activity

Figure 9. Sequencing results. Sequencing results showed an indel mutation in exon 2 (-40bp/+38bp) from the other allele of Hbb-b1 in clone A.

3.4 evaluation of potential off-targets

Based on BLAST results and the presence of the PAM sequence in the vicinity of the gRNA target site, only one non-specific cleavage site was identified in exon regions for both gRNA 3 and 4 (Hbb-b2). The PCR product related to this gene was evaluated at the length of pb966 under T7E1 enzyme nuclease activity.

The presence of only one band similar to the length of the PCR product related to normal ESC genomic DNA as a control confirmed the absence of unwanted mutation in this gene region. (Figure 10).

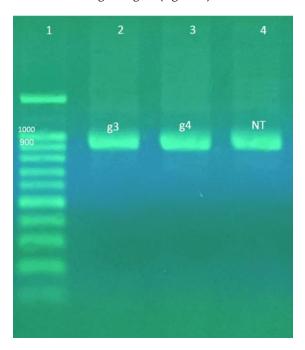


Figure 10. Evaluation of non-targeted cleavage regions of gRNA 3 and 4, well 1 in the form of a 100bp molecular weight marker.

4 Discussion

The purpose of producing laboratory models and thalassemic mice is to understand the mechanisms involved in the disease and to find therapeutic strategies to treat or reduce the complications of the disease. In a review study conducted in 2015, the types of mouse models with thalassemia that have been used so far were introduced(5). Until now, beta-thalassemia mouse models have been generated using older editing systems based on restriction enzymes (6). These methods are very time-consuming and require many repetitions.

The main goal of treatment in patients with thalassemia major is to reduce anemia without frequent blood transfusions and prevent the complications of ineffective erythropoiesis (7). L. C. Skew and colleagues used ethylnitrosourea exposure to create a 3.3 kb fragment deletion in the mouse β -globin regulatory region to create an animal model of beta-thalassemia. Further investigation showed that in the case of the initial presence of a mutation in mice, this exposure could cause its occurrence and increase (3). Dominic J and his colleagues presented Hbb th3/- thalassemia model by removing a 16-kb fragment including both β -minor and β -major genes in mouse embryonic stem cells and transferring this mutation to germ cells in mice. The deletion was done with the help of a vector containing two restriction enzymes, HindIII and BamHI (8). Aoli Yang and colleagues designed and presented another β 0 thalassemia mouse model using the "Plug and Socket" system. In this model, a 20 kb fragment containing two β -minor and β -major genes of adult rats was deleted (4).

Over the past decade, several studies have used efficient and precise CRISPR/Cas9 systems in human and mouse primary hematopoietic progenitor and stem cells to identify therapeutic targets by examining the functional properties of gene products and disease modeling. For example, in 2014, Felix C. Giani and his colleagues specifically inactivated SH2B3 in human pluripotent stem cells with the help of the CRISPR/Cas9 genome editing system, which led to the stimulation of more proliferation of erythroid cells while maintaining differentiation, increasing maturity and general function of red blood cells derived in laboratory conditions (9). By using this system, it is possible to achieve disease-specific phenotypes with acceptable efficiency by knocking out a specific gene in healthy iPS cells (10). Also, many studies have been conducted on point mutation repair using template-mediated repair (11, 12).

Hbb th1 /th1 mice (homozygous deletion of βmajor gene) and Hbb th3 / + (heterozygous deletion of both βminor and βmajor genes) show the beta-thalassemia intermedia phenotype in humans (anemia, abnormal erythrocyte morphology, iron overload, and splenomegaly) (7) and have been used in research in recent years(13, 14, 15, 16, 17). For example, in a study conducted in 2014, Suragani and his colleagues showed that RAP-536, as an inhibitor of the Smad2/3 signaling pathway, led to a reduction in IE, followed by a reduction in disease pathology in thalassemic model mice (Hbbth1/th1 and Hbbth1/th1 intermediate thalassemia mouse models). becomes Hbbth3/+)(16).

Beta-thalassemia mouse models need to maintain the expression of some β -globins for survival and therefore show characteristics similar to those observed in intermediate beta-thalassemia patients (β +) (18). Although a model of beta thalassemia major (β 0) has also been produced temporarily through surgical techniques (19).

Considering the lack of expression of fetal globins in an adult mouse, the expression of Hbb-b2 in them shows a role like the fetal-like β -globin gene (γ -globin in humans), and it can increase up to 2.5 times compared to its regular expression in the condition of non-expression of Hbb-b1, which leads to maintaining the normal ratio of α/β synthesis and reducing the complications of the disease (3, 20).

After the erythroid differentiation process, a greater expression of Hbb-b2 was seen in the Hbb-b1 +/ and Hbb-b1 -/-groups compared to the wild-type form. Competition between mature -globins at the transcription level at the -globin locus is causing this altered expression of Hbb-b2, according to the last mouse thalassemia models. The higher expression of minor β -globin is the compensatory mechanism of cells to reduce (in Hbb-b1 +/- group) or lack of expression of significant β -globin (in Hbb-b1 -/- groups) in these cells.

The final vision of this research was to produce a laboratory model of beta-thalassemia using the CRISPR/Cas9 method based on the results of this study. For this purpose, specific gRNAs were designed to identify and target the Hbb-b1 gene. Moreover, the expression of Hbb-b1 in one or both alleles of ES cells was knocked out using the CRISPR/Cas9 system.

5 Conclusion

The change made on the β-globin gene in ESCs allows a laboratory thalassemic model to be available. In addition, the results of this study provide an opportunity to generate an intermediate thalassemia mouse model (Hbb th1/th1 mouse) in a shorter time using an efficient and accurate genome editing system compared to the conventional thalassemia mouse generation method. Evaluation of treatment methods in thalassemia, including gene therapy to increase the efficiency of beta or gamma hemoglobin and identify and reduce ineffective erythropoiesis-reducing factors in vitro studies along with the use of mouse models (with the possibility of evaluating the effect of the methods used on the index blood disorders, reduction of ineffective erythropoiesis, iron overload, etc.) will lead to the development of suitable treatments for this disease.

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