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CRISPR/CAS9 MEDIATED GENE THERAPY TO CORRECT β-THAL PATIENT-SPECIFIC INDUCED PLURIPOTENT STEM CELLS AS A THERAPEUTIC STRATEGY FOR β -THALASSEMIA IN ASIA

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Abstract

Beta thalassemia is a common genetic is order caused by point mutations or small deletions in the β-globin (HBB) gene, leading to functionally defective Hemoglobin (Hb).Currently, patients retreated with hematopoietic stem cell transplantation as a curative treatment, of which the scope is limited due to the difficulty in finding human leukocyte antigen (HLA)matched healthy donors and allow-immune response. Here we discuss a permanent cure for the disease employing the novel high precision genome editing technique; CRISPR-Cas9 to precisely correct the HBB mutations in the β-thal patient specific iPSCs (induced pluripotent stem cells). Healthy hematopoietic stem cells (HSCs) generated in vitro from the corrected iPSCs can be used for transplantation to restore the normal function of RBCs. We have focused on the frequently occurring HBB mutations in the Asian population responsible for β-thalassemia and suggested the potential patient dependent mutational targets for CRISPR/Cas9mediated genetherapy.Wehavetabulatedthepotentialmutation-specificCRISPR/Cas9guideRNA (gRNA) for targeted editing of the respective mutations. Based on the target sequence there pair donor template DNA can be designed to correct any of the target mutations in the discovered loci through homologous recombination to restore the correct sequence of HBB. A complete proposed treatment method employing the above strategy has been illustrated, which could be implemented to permanently eradicate β-thalassemia from the Asian region, as well as the world wide population.

Keywords: Betathalassemia, CRISPR/Cas9, Homologousrecombination, iPSC, Gene therapy, β-Globingene.

Introduction

Molecular Basis of Beta Thalassemia

Beta thalassemia is one of the most common and widely encountered genetic diseases worldwide[1–3]. It is mainly caused by point mutations or small deletions leading to frameshift mutations in the β-globin (HBB) gene which codes for the β-subunit of Hemoglobin (Hb)[1,4–7]. As a result, the mRNA transcription, splicing, translation or the post translational stability of the product of the HBB gene are affected and leads to quantitative or qualitative diminution of β-globin chains[2,4,6,8]. Consequently, deficiency of functionally effective Hb will bring about moderate or severe anemic conditions[1,3,6,8–10]. The complete absence of the production of β-globin due to point/deletion mutations result in the disease condition called β-zero (B0) thalassemia, whereas the reduced output of β chains cause β-plus (B+) thalassemia[6,8].

Thalassemia is an autosomal recessive hematological genetic disorder where the disease is surfaced (thalassemia major) if both the maternal and paternal chromosomes carry diseased alleles with mutations (homozygous state). An individual carrying only one diseased allele will have the clinically silenced phenotype (thalassemia minor) and hence is a carrier. An individual can be a compound heterozygote if he/she carries two different types of mutations in the two alleles[1,8,9].

Hb is the major oxygen transporter protein that delivers oxygen from lungs to peripheral tissues and carbon dioxide to lungs. It is an iron containing tetramer protein composed of two α-globin and two β-globin chains. In the developing state of erythrocytes, α and β-globin subunits associate to form a stable hetero-dimeric Hb[5]. The occurrence of mutations in one or both copies of the HBB gene will alter the quality or the quantity of the β-globin[1–3,6] and creates an imbalance of α and β-globins as the synthesis of structurally and functionally normal α-globins are continued. Consequently, excess α-globin will be precipitated and leads to excessive destruction of red blood cells (RBCs) and a series of physiological complications[5].

Current Therapies Available For Beta thalassemia

There have been persistent records on the occurrence of β-thalassemia in Asia[1,3,7,8]. Over the recent years, increasing numbers of patients have been recorded with severe forms of β-thalassemia and the frequency of carrier genotypes remains undiminished over populations. For example,in Sri Lanka, an estimated ~ 0.5 million β-thalassemia carriers have been discovered[7,8,10,11]. Therefore, the disease is posing a critically important public health problem for the region. The prevalence of many carrier genotypes have been reported so far with diverse mutations on different loci in the HBB gene[1,3,7,8,10].

The major problematic consequence of β-thalassemia is the severe anemia and iron overload[4,6]. The current therapies include regular and intermittent blood transfusion to provide necessary Hb and chelation therapy to remove excess iron in the blood[8]. These treatments should continue throughout the life time if the patient is a thalassemia major. Up to date, these are the only solutions for the complications brought about by the disease and are not curative measures for the disease. Critical complications with the blood transfusion include progression of infections [Hepatitis A, B and C, Human Immunodeficiency Virus (HIV), Staphylococcus aureus and parvovirus] and allo-immunization (development of one or more anti-red blood cell antibodies in the host)[3].

Hematopoietic stem cell transplantation is another popular approach to treat thalassemia as it permanently provides a healthy population of stem cells to continuously generate non-thalassemia RBCs[4,6]. However, stem cell therapy is challenging due to the difficulty in finding human leukocyte antigen (HLA) matched healthy donors, adverse effects caused by graft versus host disease, viral contaminations (HBV, HCV) and immunoreactions[4,6]. Another stem cell-based therapy is to manipulate embryonic stem cells to correct the defect in HBB gene. Due to the ethical issues in manipulating human embryonic stem cells, application of this method also remained in question for some period.

Recent Advances in Gene Therapy on Patient Specific Ipscs

The best remedy for the disease is to correct the mutations in the patient specific iPSCs (induced pluripotent stem cells). IPSCs are undifferentiated cells like pluripotent stem cells and embryonic stem cells. They can self-renew and potentially differentiate into hematopoietic stem cells (HSCs), hematopoietic progenitor cells and mature hematopoietic cells in an appropriate culture system[4].

The corrected iPSCs are capable of generating healthy hematopoietic stem cells to produce normal, functional red blood cells. Since the modified stem cells are patient specific, iPSC therapy can overcome the immune challenges associated with allogenic transplantation and presents a solution for the ethical and practical challenges regarding manipulation of embryonic stem cells[4,6,9,12]. The correction of mutations in the iPSCs can be achieved through genetic engineering using homologous recombination[4,6,12]. High precision gene editing by targeted nucleases can be conjointly employed with homologous recombination as a powerful tool for targeted gene editing with high accuracy and efficiency[4,6,9,13–16].

Since many years, the gene editing has been done using targeted nucleases as molecular scissors via several approaches, such as ZFNs (zinc finger nucleases), and TALENs (transcription activator like effector nucleases)[14,17,18]. These technologies were developed using the principle of DNA protein recognition and specific binding. The DNA binding domains of the above systems needs to be target specific. Thus, the difficulties of protein designing, modelling, synthesis and validation have become a barrier to this engineered nuclease for routine use[14]. Recently, a new frontier of genome editing with CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats/ CRISPR-associated-9) was discovered to initiate precise and easy gene editing, with no limitations by organism. This can be easily targeted to any new DNA sequence by simply designing a pair of oligos encoding the 20-base pair guide RNA sequence[9,12,15,16,18,19].

Mutation Correction in iPSCs with CRISPR/Cas9

CRISPR/Cas9 is a microbial acquired immune system, which uses RNA guided end nuclease to cleave invaded foreign genetic material. It is a programmable sequence specific end nuclease, which has the ability to precisely edit endogenous genomic loci and has been used for the correction of most of the genetic diseases[6,9,12,13,16,18,19]. The CRISPR/Cas9 system has two main components, a guide RNA (gRNA) and Cas9, which is an RNA- guided endonuclease. Guide RNA is a chimera of crRNA (CRISPR RNA) and tracr RNA (trans-activating crRNA). CrRNA is a 20 bp stretch of nucleotides that recognizes the target sequence by complementary base pairing where a stracr RNA provides the binding site for Cas9 nuclease. Precise binding and cleavage of the target DNA sequence by Cas9 relies on the PAM (photo spacer adjacent motif) sequence. PAM is a 3-base pair DNA sequence (NGG for Cas9) that must be available at the target site for Cas9 to precisely create a double stranded break in the target DNA sequence. Once the PAM is identified by Cas9, the hybridization of cr RNA with target region occurs and the double stranded break is generated by Cas9 nuclease, precisely 3 bases upstream of the PAM. This double stranded break can either be repaired by NHEJ (non-homologous end joining) pathway, an error prone repair mechanism which often create gene inactivation mutations by formation of indels or by HDR (homology directed repair), a defined precise repair by providing an exogenous homologous donor template (Figure 1)[20, 17].

CRISPR/Cas9 mediated HDR modifications have been widely used as an approach to correct mutations associated with genetic disorders in mammalian cells[6,9,12,13,16–18]. For example, CRISPR/Cas9 system has been delivered through intramuscular, intraperitoneal or intravenous injection to correct the Duchenne Muscular Dystrophy in mouse models by

targeting responsible exon, intron regions of the Dystrophin gene to successfully correct targeted mutations[18]. The efficacy of CRISPR/Cas9 mediated HDR for in vivo gene therapy in mouse models of human hereditary liver diseases has been proven by recent studies[18]. Researchers have explored the potency of CRISPR/Cas9 in disease therapy in vitro, in vivo and ex vivo to address many other genetic disorders such as Retinitis pigmentosa (correction of Rhodopsin gene mutation)[18] and Cystic Fibrosis (correction of the deletion in Cystic Fibrosis Tran membrane Regulator gene)[21]. Recently, CRISPR/Cas9 has been used to correct sickle cell anemia[21] and thalassemia[6,9] in human iPSCs. Correction of the mutations associated with thalassemia in patient-specific iPSCs can be done by CRISPR/Cas9 followed by HDR and the corrected iPSCs can be used for transplantation to restore the normal function of RBCs. Xiaohua Niu and coworkers (2016) have corrected patient specific β-thaliPSCs by CRISPR/Cas9, transplanted into mice models and the functional HBB expression has been successfully observed in mice without any tumorigenic potential[6,12]. Based on the same principle, we have focused on suggesting an implementable approach to treat β-thalassemia in Asian population. Here, we describe a method to correct HBB mutations in β-thaliPSCs by employing a mutation-specific CRISPR/Cas9 gRNA and single stranded donor oligonucleotides (ssODNs) to repair the mutations[22]. However, this is a personalized treatment method since it primarily depends on the genetic composition and the mutations present in the diseased individual. The implementation of the above curative approach to treat β-thalassemia necessitates the knowledge of the mutations/polymorphisms in the HBB gene responsible for the disease in each individual.

Mutation Specific gRNA and ssODN to Correct HBB mutations

Up to date, many genetic mutations have been discovered that are responsible for the different types of β-thalassemia that are found in Asia[1,3,7,8,10]. We have cumulated published information on many HBB mutations responsible for β-thalassemia discovered worldwide and refined the frequently occurring HBB mutations in the gene pool of the Asian population, sorted by region. Table 1 consists of the mutations that are found in high frequencies in the Indian subcontinent. The listed mutations were spotted in patients with the clinical diagnosis of β-thalassemia major or intermedia (β-thalassemia in which the clinical severity of the disease is somewhere between the mild symptoms of the β-thalassemia minor and the severe manifestations of β-thalassemia major)attending clinics throughout the region[1,3,7,8,10]. Figure 2(a) depicts the structure of the HBB gene and Figure 2(b) illustrates the loci and the respective mutations frequently positioned on the HBB gene sequence in β-thal patients found in the region[1–3,7,8,10]. Several of the listed mutations occur around intron, exon splicing sites (IVS), hence generating splice variations leading to undesired alternations in the HBB transcripts, causing changes in the β-globin. Most of the mutations occur in the coding region of the gene, directly altering the HBB transcripts and the final protein product[2,7,8].

Built on our analysis of published data, HBB mutations that occur in high frequency were identified as potential targets for CRISPR/Cas9 mediated gene therapy in β-thal patients. Based on the identified mutations, we have designed specific CRISPR gRNAs to target the patient-specific mutations in HBBloci and correct them using CRISPR gene editing.Table 1 describes the loci, position of the mutations and the designed, possible gRNAs which can be used for targeted editing. (We have only focused on the base changes in the forward strand of HBB.)As illustrated in Table 1, it is verified that a vast majority of the subjective HBB mutations can be targeted by a universal CRISPR gRNA, due to the close proximity of mutations to the gRNA target site. Consequently, all the mutations can be targeted using one of the two universal gRNAs. As illustrated in Figure 2(b), majority of the HBB mutations occur within ~200 bpconserved region. On that account, it manifests a possibility where a single common ssODN carrying the correct gene sequence can be used to correct any of the constricted mutations depicted in Figure 2(b). The repair donor templates should carry the correct sequence which eventually replaces the target region including mutations and restore the normal gene function. The designed universal ssODN and gRNA sequences should be experimentally evaluated for their specificity, efficacy, and most importantly for their off-target effects.

Proposed Treatment method

Figure 3 illustrates the proposed implementable treatment method. As the initiative, the mutations on the HBB gene of the patient has to be discovered using appropriate techniques such as PCR (polymerase chain reaction) and DNA sequencing. Specific guide RNA must be designed which recognizes the patient-specific mutation/s. Since majority of the mutations occur in a confined region with close proximity, a universal gRNA can be used to target the mutations. (The proposed universal RNA sequences, targeting frequently occurring HBB mutations are listed in Table 1.) Next, the gRNA sequence must be cloned into a suitable CRISPR plasmid vector compatible for human (e.g. pSpCas9 (BB)-2A-puro), which carries a cas9 gene for co-expression with the gRNA. Similarly, compatible, specific ssODN carrying the correct sequences to replace the mutation has to be designed for direct transfect ion along with the recombinant CRISPR plasmid. Since majority of the mutations occur in a constricted region of the gene, (Figure 2(a) and (b)) a universals ODN corresponding to the mutation-rich region can be used in many casesas the target cells, iPSCs generated from the diseased individual must be used. Therefore, patient derived somatic cells must be grown in appropriate culture conditions to differentiate into iPSCs. Next, the gene correction can be carried out invitro using the designed CRISPR/Cas9 system (cloned with target specific gRNA) and the

donor template ssODN to correct the mutation. Following the modification, clonal selection must be carried out (e.g. through antibiotic selection using the marker genes in the CRISPR plasmid) to select the successfully transected clones. After selection, the correction of the mutations must be validated by amplifying the modified region followed by DNA sequencing. The accurately modified iPSCs must be differentiated into HSCs invitro by providing necessary culture conditions. Finally, the corrected patient specific HSCs can be transplanted to patient's bone marrow. These corrected HSCs themselves can permanently produce normal, functional RBCs expressing the accurate β-globin subunit of the Hb. As a result, the potential to express β-globin in normal quantities/quality will be acquired and restored by the normal RBCs[9, 12].

Concluding Remarks And Future perspectives

As demonstrated in the Figure 3, we have proposed an efficient method to correct β-thalassemia associated genetic mutations in Asian population. We have illustrated the potential patient dependent mutational targets for CRISPR/Cas9 mediated HDR modifications by considering the frequently occurring HBB mutations found in the gene pool of the Asian population1–3,7,8,10. We have also discussed the possibility of using universal RNAs and repair donor templates to correct any of the mutations in the discovered loci of the HBB gene (Table 1).However, the off-target effect of these sequences remain to be experimentally evaluated.

After evaluating the efficacy and specificity of gRNA and ssODN sequences, a β-thal specific CRISPR tool kit consisting of a universal gRNA cloned into a suitable CRISPR vector (e.g. pSpCas9(BB)-2A-puro), along with a universal ssODNcan be designed to correct the frequently occurring HBB mutations in β-thal patients.

In contrast to the conventional treatment methods used against β-thalassemia, the proposed method provides a profound, permanent and effective cure for the disease[6,9,12]. Moreover, since the disease correction is done in patient specific iPSCs, the proposed strategy is devoid of adverse effects due to host, graft mismatches and immune reactions. Since the correction is done invitro, it provides the ease of using mainstream transfection methods (e.g.lipofectamine/cationic polymer mediated transfection) to introduce the exogenous DNA[22]. The markedly proven efficiency and efficacy of CRISPR/Cas9 mediated HDR systems to correct mammalian genomes[16,18] signifies this strategy to have a promising potential. The capability of enhancing the specificity of gene targeting by the CRISPR/Cas9 system through precise and accurate designing of gRNA and ssODN, thereby minimizing off-target effects and toxic effects, and the convenience of operation[6,15,16,18,19] profoundly marks this strategy advantageous as a safe curative approach for β-thalassemia..

However, practical implementation of this proposed treatment method in wide scale necessitates overcoming a few hurdles. Since this is a personalized disease treatment strategy, unlike mass drug production, patient specific gRNA and ssODN may have to be designed separately.Screening for off target mutagenesis and analyzing toxicity of the CRISPR/Cas9 system complemented with the ssODN have to be thoroughly evaluated as untargeted CRISPR/Cas9 editing and homologous recombination can bring about much complex genetic deffects. Therefore, such effects should be proven minimal in order to be implemented as a treatment strategy. In each case, the designed sequences should be proven to work in practice with acceptable accuracy and efficiency prior to application. In addition, it requires DNA sequencing at multiple stages and growth/ maintenance of patient specific iPSCs in culture. Hence the economic aspect of implementing this strategy remains a challenge. In spite of that, this proposed treatment method holds an enormous promising potential as a reliable, convenient and permanent therapeutic strategy to eradicate β-thalassemia in the Asian region as well as worldwide.

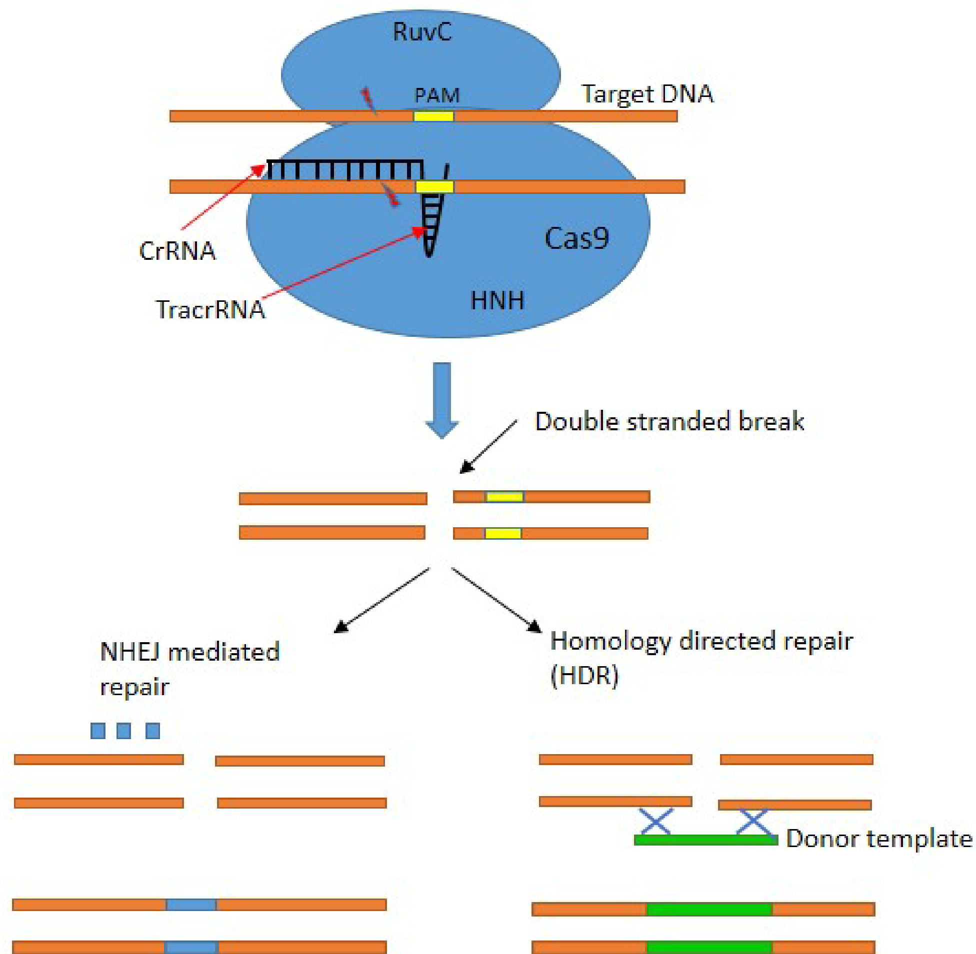
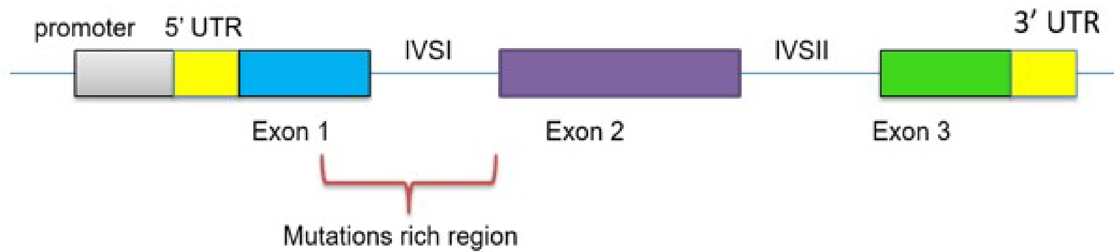


Figure 1: CRISPR/Cas9 mediated gene editing by target DNA cleavage and repair.

The Cas9 endonuclease is guided by the target specific CrRNA to the target DNA. A double stranded break (DSB) is generated by the two active domains of Cas9 (HNH and RuvC), precisely 3 bp upstream of the PAM at the target region. The DSB is repaired by one of the two methods; homology directed repair (HDR) using a donor template with homologous regions to the target locus or the non-homologous end joining (NHEJ) method where nucleotides are randomly incorporated to seal the DSB, generating indel mutations.

(a)



(b)

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-35   GGGCATAAAAGTCAGGGCAGAGCCATCTATTGCTTACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCATCTGACT
 66   CCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACA
166   GGTTTAAGGAGACCAATAGAAACTGGGCATGTGGAGACAGAGAAGACTCTTGGGTTTCTGATAGGCACTGACTCTCTCTGCCTATTGGTCTATTTTCCCA
266   CCCTTAGGCTGCTGGTGGTCTACCCTTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAACCCTAAGGTGAA
366   GGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAACCTCAAGGGCACCTTTGCCACACTGAGTGAGCTGCACTGTGAC
466   AAGCTGCACGTGGATCCTGAGAACTTCAGGGTGAGTCTATGGGACGCTTGATGTTTTCTTTCCCCTTCTTTTCTATGGTTAAGTTCATGTCATAGGAAGG
566   GGATAAGTAACAGGGTACAGTTTAGAATGGGAAACAGACGAATGATTGCATCAGTGTGGAAGTCTCAGGATCGTTTTAGTTTCTTTTATTTGCTGTTCAT
666   AACAATTGTTTTCTTTTGTTTAATTCTTGCTTTCTTTTTTTTTCTTCTCCGCAATTTTTACTATTATACTTAATGCCTTAACATTGTGTATAACAAAAGG
766   AAATATCTCTGAGATACATTAAGTAACTTAAAAAAAAACTTTACACAGTCTGCCTAGTACATTACTATTTGGAATATATGTGTGCTTATTTGCATATTCA
866   TAATCTCCCTACTTTATTTTCTTTTATTTTTAATTGATACATAATCATTATACATATTTATGGGTTAAAGTGTAATGTTTTAATATGTGTACACATATTG
966   ACCAAATCAGGGTAATTTTGCATTTGTAATTTTAAAAAATGCTTTCTTCTTTTAATATACTTTTTTGTTTATCTTATTTCTAATACTTTCCCTAATCTCT
1066  TTCTTTCAGGGCAATAATGATACAATGTATCATGCCTCTTTGCACCATTCTAAAGAATAACAGTGATAATTTCTGGGTTAAGGCAATAGCAATATCTCTG
1166  CATATAAATATTTCTGCATATAAATTGTAACTGATGTAAGAGGTTTCATATTGCTAATAGCAGCTACAATCCAGCTACCATTCTGCTTTTATTTTATGGT
1266  TGGGATAAGGCTGGATTATTCTGAGTCCAAGCTAGGCCCTTTTGCTAATCATGTTCATACCTCTTATCTTCCTCCCACAGCTCCTGGGCAACGTGCTGGT
1366  CTGTGTGCTGGCCCATCACTTTGGCAAAGAATTCACCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTGTGGCTAATGCCCTGGCCCACAAG
1466  TATCACTAAGCTCGCTTTCTTGCTGTCCAATTTCTATTAAAGGTTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATATTATGAAGGGCCTTGAG
1566  CATCTGGATTCTGCCTAATAAAAAACATTTATTTTCATTGC
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Figure 2:
(a) Schematic representation of human HBB gene with the promoter and three exons denoted by boxes. The mutation rich region occurs around the first intron, exon splicing site IVSI. (HBB promoter, exon 1,2 and 3 are indicated in gray, blue, purple and green respectively.) (b) Sequence of the genomic HBB gene locus highlighting the common mutations (red) found in Asia. (Exon 1,2 and 3 are indicated in blue, purple and green respectively.)

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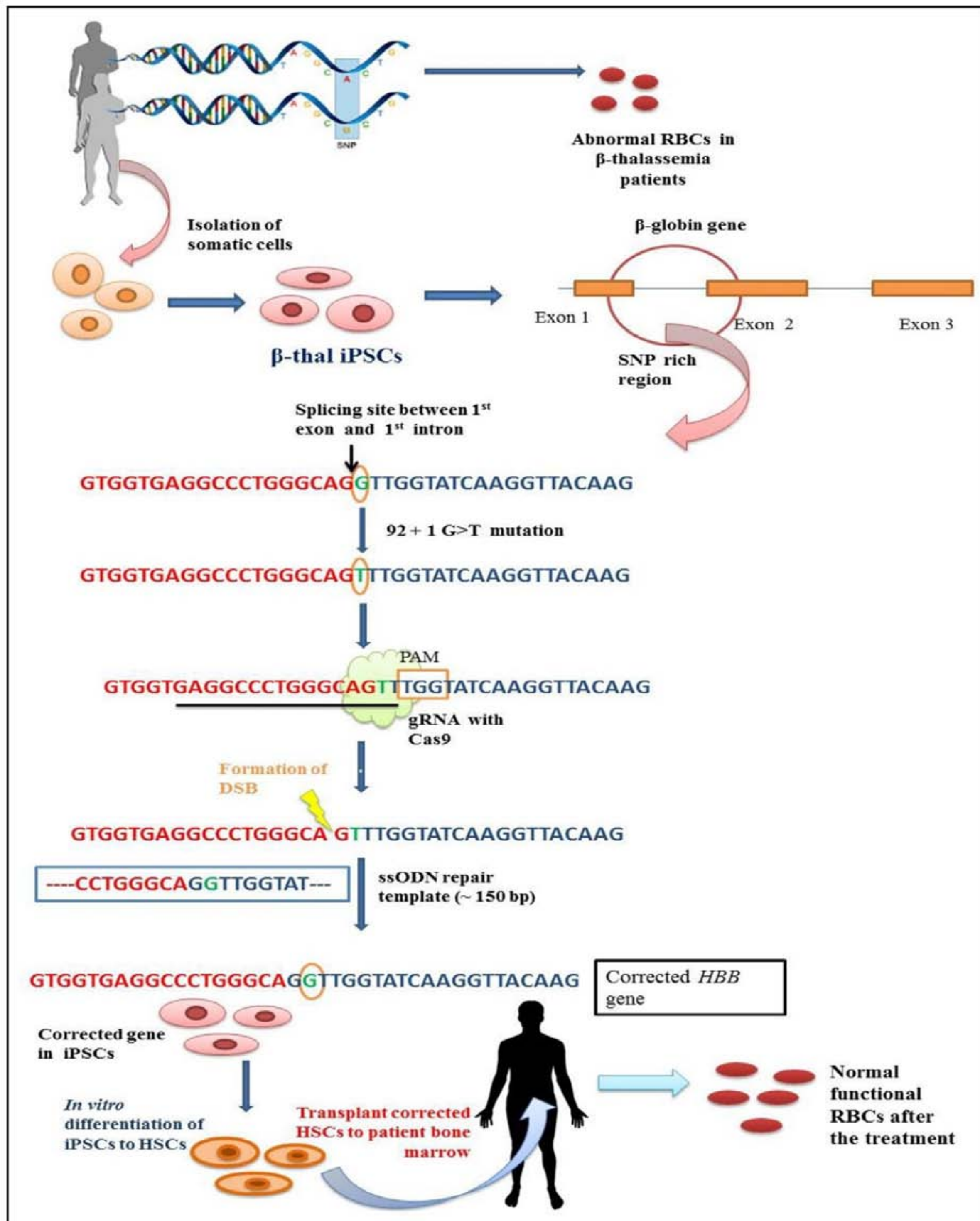

Figure 3: Proposed therapeutic strategy to correct β-thalassemia in patient derived iPSCs. The HBB mutation/s in the β-thal patient specific iPSCs is corrected by CRISPR/Cas9 mediated gene editing with target specific gRNA, followed by ssODN mediated HDR to restore the correct sequence. The corrected iPSCs are differentiated in vitro into HSCs with the ability to produce normal RBCs. Generated HSCs are transplanted into patient's bone marrow to permanently produce normal, functional RBCs.

Table 1: The common mutations on the HBB gene found in the Asian region that are responsible for different types of β-thalassemia, proposed 20 nucleotide crRNA (PAM is highlighted in red)targeting the forward strand to correct the respective mutations, by CRISPR/Cas9 system. (* South Asia, €Thailand, # Middle East, ± China)

Mutation	Locus	position	Guide RNA
A→G * ± €	C.-78	-28	GGTAGACCACCAGCAGCCTAAGG
G→C*	IVSI-130	13	GTCTGCCGTTACTGCCCTGTGGG
G→ A*	IVSI-130	13	GTCTGCCGTTACTGCCCTGTGGG
A→C*	IVSI-129	14	GTCTGCCGTTACTGCCCTGTGGG
-CT*	CD5	67/68	GTCTGCCGTTACTGCCCTGTGGG
-13 bp*	CD6/CD10	69-83	GTCTGCCGTTACTGCCCTGTGGG
A→T*	HbS CD 6	70	GTCTGCCGTTACTGCCCTGTGGG
+**G** * ±	CD 8/9	77/78	GTCTGCCGTTACTGCCCTGTGGG
-T*	CD 15	96	GTCTGCCGTTACTGCCCTGTGGG
G→A*	CD 15	97/98	GTCTGCCGTTACTGCCCTGTGGG
-C*	CD 16	101	GTCTGCCGTTACTGCCCTGTGGG
G→A*	CD 26	129	GTCTGCCGTTACTGCCCTGTGGG
G → C*	IVSI-5	138	GTCTGCCGTTACTGCCCTGTGGG
G → A*	IVSI-1	142	GTCTGCCGTTACTGCCCTGTGGG
G → T*	IVSI-1	142	GTCTGCCGTTACTGCCCTGTGGG
G →A* #	C.92+1	143	GTCTGCCGTTACTGCCCTGTGGG
G→C* # €	C.92+5	147	GTCTGCCGTTACTGCCCTGTGGG
-TCTT * ±	CD 41/42	305-308	GGTAGACCACCAGCAGCCTAAGG
-CTTT*	CD41/42	306-309	GGTAGACCACCAGCAGCCTAAGG
-A*	CD 55	346	GGTAGACCACCAGCAGCCTAAGG
G→A*	IVSII-1	496	GGTAGACCACCAGCAGCCTAAGG

Competing interests: The authors declare that they have no competing interests.

Author's contributions: OJ and MS equally contributed to the work and wrote the manuscript. All authors read and approved the final manuscript.

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