Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 345 (2006) 14–20
BBRC
www.elsevier.com/locate/ybbrc

A novel single step double positive double negative selection strategy for β-globin gene replacement

H. Khanahmad [a], M.R. Noori Daloii [b], M.A. Shokrgozar [c], K. Azadmanesh [d], A.R. Niavarani [a,1], M. Karimi [a], B. Rabbani [a], M. Khalili [a], R. Bagheri [a], F. Maryami [a], S. Zeinali [a,*]

[a] *Biotechnology Research Centre, Pasteur Institute of Iran, Tehran, Iran*
[b] *Department of Medical Genetic, Faculty of Medicine, Tehran University of Medical Science, Tehran, Islamic Republic of Iran, Tehran, Iran*
[c] *National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran*
[d] *Department of Hepatitis and AIDS Pasteur Institute of Iran, Tehran, Iran*

Received 5 April 2006
Available online 25 April 2006

Abstract

β-Thalassemias are a heterogeneous group of autosomal recessive disorders, characterized by reduced or absence of the β-globin chain production by the affected alleles. Transplantation of genetically corrected autologous hematopoietic stem cell (HSC) is an attractive approach for treatment of these disorders. Gene targeting (homologous recombination) has many desirable features for gene therapy due to its ability to target the mutant genes and restore their normal expression. In the present study, a specific gene construct for β-globin gene replacement was constructed consisting of: two homologous stems including, upstream and downstream regions of β-globin gene, β-globin gene lying between hygromycin and neomycin resistant genes as positive selection markers and thymidine kinase expression cassettes at both termini as negative selection marker. All segments were subcloned into pBGGT vector. The final plasmid was checked by sequencing and named as pFBGGT. Mammalian cell line COS-7 was transfected with linear plasmid by lipofection followed by positive and negative selection. DNA of the selected cells was analyzed by PCR and sequencing to confirm the occurrence of homologous recombination. In this novel strategy gene replacement was achieved in one step and by a single construct.

Keywords: β-Thalassemia; Gene therapy; Homologous recombination, Gene replacement

β-Thalassemias are a heterogeneous group of autosomal recessive disorders, characterized by reduced (β^{+}) or absence (β^{0}) of the β-globin chain production. The severity of β-thalassemia is directly linked to the degree of imbalance in the production of α- and β-globin chains [1]. Almost 200 β-thalassemia mutations have been characterized so far. These mutations can affect transcription, mRNA splicing, expression, and stability of beta globin chain after translation [1]. β-Thalassemia is most prevalent in the Mediterranean region, the Middle East, the Indian subcontinent, and south East Asia. Patients with this disorder have ineffective erythropoiesis, hemolytic anemia, hypochromic microcytic anemia, and hepatosplenomegaly. Regular transfusion in these patients leads to iron overload and must be treated by chelating agents [1].

At present, the only available curative therapy is allogenic bone marrow transplantation. This therapeutic option is not available to the majority of patients, in the absence of suitable donor, and has immunological complications such as graft versus host disease (GVHD) [2,3]. Human hematopoietic stem cells (HSC) have the potential to regenerate the entire hematopoietic system and are attractive targets for gene therapy. Transplantation of genetically corrected autologous HSC is an attractive approach for the cure of these disorders [4,5].

* Corresponding author. Fax: +98 21 66480780.
E-mail address: siruszeinali@yahoo.com (S. Zeinali).
[1] Current address: Hematology, Oncology and BMT Center, Tehran University of Medical Sciences, Tehran, Iran.

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.04.060

Different approaches have been tried for gene therapy of β-thalassemia so far, most of which have used viral vectors [6,7]. Gene delivery by viral vectors is highly efficient but has some disadvantages which limit their applications [8]. The capacity of these vectors is usually not enough for inserting the gene of interest and its tissue-specific sequences such as locus control region (LCR) of β-globin gene [9]. Inserting the mini LCR in these vectors incorporated with β-globin gene could increase the expression level of the β-globin gene, but the variable expression level, low titer, transcriptional silencing, and instability of the integrated β-globin gene also limit the application of the viral vectors in β-thalassemia gene therapy [10–12]. Retroviral vectors integrate the inserted gene randomly into the genome and this random integration can activate oncogenes or inactivate essential genes [13]. There is a possibility for producing wild type or replication competent retrovirus during assembling retroviral vectors [8,14]. According to mentioned problems with viral vectors, some approaches for gene therapy have focused on gene targeting or gene replacement whereby the defective gene is removed and a normal gene is inserted at the same position in the chromosome without any changes in genome.

Normal HSC has one copy of β-globin gene on chromosome 11 and two copies of α-globin gene on chromosome 16; therefore, there is an α4/β2 gene ratio. This ratio is essential for normal expression of α and β, and subsequent assembly of normal hemoglobin. In gene therapy by viral vectors the copy number of the gene which is integrated is not controllable [15,16], but in gene targeting the copy number of targeted gene will remain intact. The molecular process controlling gene replacement in gene targeting is homologous recombination (HR) [17] which occurs at a very low frequency in mammalian cells thereby limiting its application in gene therapy [18].

In the present study, a novel construct was designed for targeting β-globin gene and the possibility of homologous recombination was evaluated in a mammalian cell line model.

Materials and methods

Plasmids and reagents. All plasmids were amplified by transforming *Escherichia coli* Top10F' cells following standard protocols [19]. In a few cases when methylation sensitive restriction enzymes were used (e.g., *Cla*I), *Dam*$^{-}$ *E. coli* strain JM 110 (Stratagene, USA) was the bacterial host. All transformed bacteria were grown in LB-Broth medium (Merck, Germany) supplemented with 100 μg/ml ampicillin (Roche, Germany). Molecular manipulation such as, plasmid and DNA extraction, T/A cloning, and enzymatic reactions were performed according to manufacturers' instructions. Human genomic DNA was extracted from 5 ml peripheral blood from a healthy person, using whole blood genomic DNA extraction kit according to manufacturer's protocol (Roche, Germany). All restriction enzymes and T4 DNA ligase used in this study were purchased from Fermentas (Fermentas, Ukraine).

Primers and polymerase chain reactions (PCRs). All primers were designed with appropriate restriction sites at 5′ end of each primer (Bold letters). The sequences of forward and reverse primers and product size for PCR were set as follows: thymidine kinase-1(1630 bp), TK1-F 5′-**GCT AGC** CAC TAT CTT GTC ACC CGG AG-3′ and TK1-R 5′-**GGT ACC** TCG ACA GAG TGC CAG CC-3′; thymidine kinase-2 (1598 bp), TK2-F 5′-**ACC GGT** AGG AGC TTC AGG GAG TGG-3′ TK2-R 5′-**CTC GAG** AGA GTG CCA GCC CTG G-3′; hygromycin resistance gene (1805 bp) HYG-F 5′-**AGA TCT** GTG GAA TGT GTG TCA GTT AGG-3′ HYG-R 5′-**GAG CTC** AGG CTT TAC ACT TTA TGC TTC-3′; neomycin resistance gene (1494 bp) NEO-F 5′-**CTT AAG** CAG GCT CCC CAG GCA G-3′ NEO-R 5′-**ATC GAT** CGA CGG GAT CCA GAC ATG-3′; human β-globin gene (2114 bp) HBG-F 5′-**GAG CTC** TAC ACT TGC AAA GGA GGA TG-3′ HBG-R 5′-**CTT AAG** CCT CCA AAT CAA GCC TCT AC-3′; upstream of HBG (2261 bp) USHBG-F 5′-**GGT ACC** CTC TGT CTC TCT CGC TGT CTC-3′ USHBG-R 5′-**ACG CGT** TCA GTT CTA AGC ATA TCT TCT CC-3′; downstream of HBG (2550 bp) DSHBG-F 5′-**ATC GAT** ACT ACC CAT TTG CTT ATC CTG-3′ DSHBG-R 5′-**ACC GGT** GAT TGT GGT ATA TGC AGT TAA GC-3′. All PCRs were performed by high fidelity Expand DNA polymerase (Roche, Germany). Typical thermal cycling program was as follows: 94 °C for 2 min, 10 cycles of 94 °Cfor 20 s, 60 °C for 30 s, 68 °C for 1 min/1 kb of length, then 20 cycles of 94 °C for 20 s, 60 °C for 30 s, 68 °C for 1 min/1 kb of length adding 20 s per each cycle, and then 15 min at 68 °C for final extension. PCR products were visualized on 1.2% agarose gel stained with ethidium bromide under UV illumination. The desired DNA bands were then cut and eluted from gel. PCR products were cloned into pTZ57T/A cloning vector (Fermentas, Ukraine) and resulting plasmids pT-NEO, pT-HBG, pT-USHBG, pT-DSHBG, pT-HYG, pT-TK1, and pT-TK2 were purified by plasmid extraction kit (Qiagen, Germany). All seven fragments were sequentially subcloned into pBGGT (GenBank Accession No. DQ384617) (Fig. 1). The final sequential order of the fragments in the construct was as follows: TK1-USHBG-HYG-HBG-NEO-DSHBG-TK2. PCRs for different fragment of the final plasmid and digestion with different enzymes were performed on pFBGGT template. This plasmid was sequenced in both directions using primers which had been designed to walk the 13.3 kb gene targeting fragment in almost 600 bp partially overlapping fragments and named as pFBGGT. Cycle sequencing process was performed by a commercial facility (Cinnagen, Iran). This latter plasmid was extracted by anion exchange chromatography (Endofree plasmid mega kit: Qiagen, Germany).

Cell culture and transfection. Biological activity of positive and negative selection markers was checked by transient transfection of Cos-7 cells (NCBI C143, National Cell Bank of Iran, Tehran, Iran) using *Xho*I linearized pFBGGT. pFBGGT was digested by *Nhe*I and *Xho*I, and digestion products were run on 1% agarose gel. The 13.3 kb band of the inserted DNA (naked DNA) was extracted from gel by gel extraction kit (Qiagen, Germany). COS-7 cells were grown in RPMI 1640 medium supplemented with 100 U/ml penicillin 100 μg/ml streptomycin, 2 mM glutamine, and 10% FCS (All from Gibco, USA), at 37 °C and 5% CO_2 in a humidified incubator. The day before transfection, 1.6×10^6 cells were seeded in 100 mm tissue culture plates. The cells were incubated until they reached 50–70% confluency and the culture medium was replaced with 7 ml of fresh medium before transfection. Cells were transfected by 4 μg of the naked DNA using Polyfect reagent (Qiagen, Germany) as instructed by the manufacturer. After 24 h, medium was replaced and 48 h after transfection, hygromycin and G418 antibiotics (both from Roche, Germany) were added to the culture at final concentrations of 150 and 600 μg/ml, respectively. This medium was changed every 3 days. 9 days after transfection, 35 μg/ml of ganciclovir was added to the culture. This latter triple treatment (hygromycin, G418, and ganciclovir) was continued for 1 week. The remaining colonies were transferred to new 60 mm dishes. One week later, the selected cells were treated with trypsin (%0.25 Gibco, USA) for 3–5 min and centrifuged at 1500 RPM for 5 min. DNA of about 600,000 cells was extracted by DNP Kit (Cinnagen, Iran) and used as template for further study by PCR.

PCR on genomic DNA samples of selected cells: PCR for TK1, TK2, hygromycin, neomycin, and the fragment which has a segment of genome above the upstream region of beta globin (USHBG) and USHBG and partial segment of hygromycin gene (This PCR was done by forward primer, SUS-F 5′-TGT GTA TCT GCG AGA GAA GTC-3′ and reverse

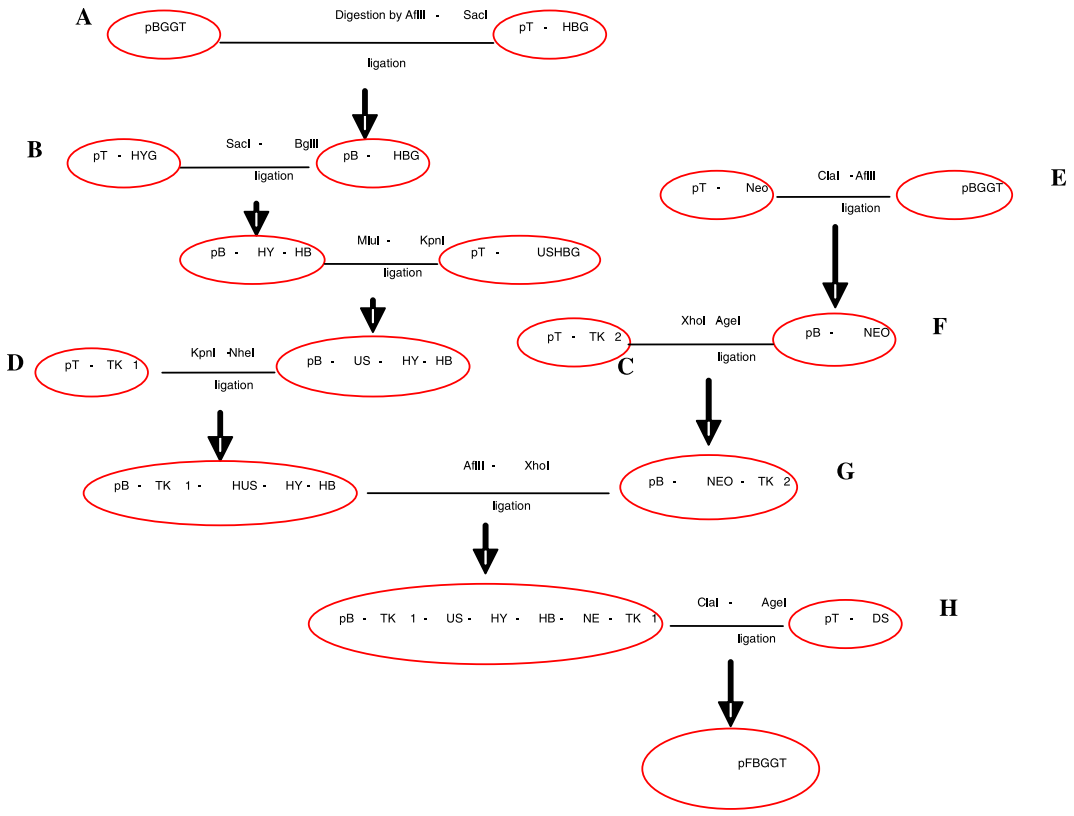

Fig. 1. Subclonings and constructing the final plasmid: pT-HBG and pBGGT were digested by *Sac*I–*Afl*II and ligated with T4 DNA ligase to making pB-HBG (A). pT-HYGRO and pB-HBG were digested by *Bgl*II–*Sac*I and the two fragments were ligated to forming pB-HYG-HBG (B). pT-USHBG and pB-HY-HB were digested by *Kpn*I–*Mlu*I and ligated to forming pB-US-HYG-HB (C). pT-TK1 and pB-US-Hy-HB were digested by *Nhe*I–*Kpn*I and ligated to form pB-TK1-US-HY-HB (D). pT-NEO and pBGGT were digested with *Cla*I–*Afl*II and ligated to forming pB-NEO (E). pT-TK2 and pB-NEO were digested by *Age*I–*Xho*I and ligated to construct pB-NEO-TK2 (F). pB-NEO-TK2 was digested by *Xho*I and *Afl*II restriction enzymes and NEO-TK2 fragment was introduced into *Xho*I–*Afl*II sites of pB-TK1-US-HY-HB to form pB-TK1-US-HY-HB-NEO-TK2 (G) This latter plasmid and pT-DSHBG were digested with *Age*I–*Cla*I and ligated, and the resulting plasmid was pFBGGT, the plasmid was the final plasmid with the following order: TK1-USHBG-HYG-HBG-NEO-DSHBG-TK2 (H).

primer, HYGRO-R4 5′-TCA GGC TTT TTC ATC ACG-3′) was performed on DNA samples. The PCR products were sequenced in both directions.

Results

Results of PCR and cloning

Seven DNA fragments were successfully amplified by PCR. Thymidine kinase complete gene was amplified from HSV-1 genome in 2 forms (TK1 and TK2). β-Globin gene and its upstream and downstream regions were amplified from human genomic DNA in 3 fragments: HBG, USHBG, and DSHBG, respectively. Neomycin and hygromycin resistance genes were amplified with their eukaryotic promoters and terminators from pRc/CMV2 and pCDNA3.1^{+}/ hygro (Invitrogen, USA), respectively (Table 1). PCR products were T/A cloned into pTZ57T/A cloning vector. All seven clones were analyzed and confirmed by digestion, PCR, and sequencing. All seven fragments were successfully subcloned next to each other to form the 13.3 kb long replacement cassette in pBGGT backbone. Amplification of related segment by PCR and restriction analysis with respective enzymes at all of the cloning and subcloning steps

Table 1
Length and specifications of each PCR product

Fragment	Specifications
TK1	1.63 kb containing promoter, gene and terminator region of HSV-I thymidine kinase
USHBG	2.2 kb upstream region of beta globin (Accession number U01317 from 59423 to 61671)
Hygro	1.8 kb, including SV40 promoter and terminator and Hygromycin resistance gene from pCDNA3.1+/Hygro
HBG	2.1 kb, promoter, coding and non-coding regions of HBG and its native terminator (Accetion number U01317 from 61877 to 63979)
Neo	1.5 kb Neomycin resistance expression cassette, from pRc/CMV2
DSHBG	2.5 kb downstream region of HBG (from 64045 to 66583)
TK2	1.6 kb including promoter, gene, and terminator of HSV-I thymidine kinase

confirmed the authenticity of these steps. pFBGGT was further checked by PCR (Fig. 2), restriction analysis (Fig. 3) and sequenced in both directions. Sequencing results confirmed lack of any known pathogenic mutation in beta globin gene and other fragments of pFBGGT. The effective dose of hygromycin, geneticin, and ganciclovir on Cos-7 cell line was already determined and the biological activity of positive and negative selection markers including, hygromycin, neomycin TK1 and TK2 was checked by transfecting Cos-7 cells with linear pFBGGT (data not shown). The results of biological activity assays of these selection markers confirmed the expression of functional proteins by these genes.

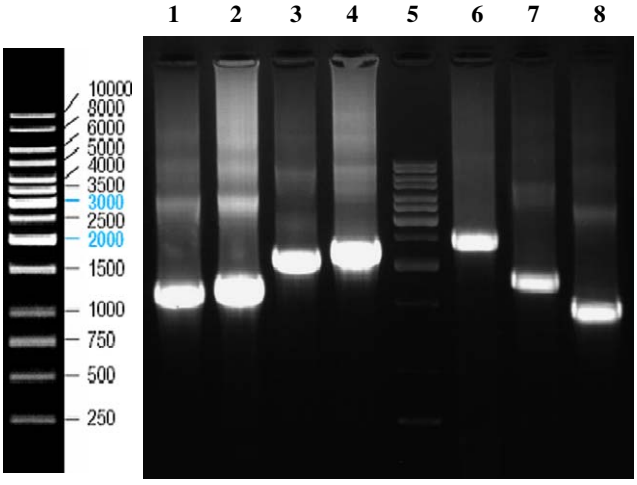

Fig. 2. PCR products of 7 fragments which were inserted in pFBGGT. Lane 1(TK2, 1600 bp), lane 2 (TK1, 1630 bp), lane 3 (HBG, 2100 bp), lane 4 (USHBG, 2200 bp), lane 5 Marker 1 kb (Fermentas), lane 6 (DSHBG, 2500 bp), lane 7 (HYGRO, 1800 bp), lane 8 (NEO, 1500 bp).

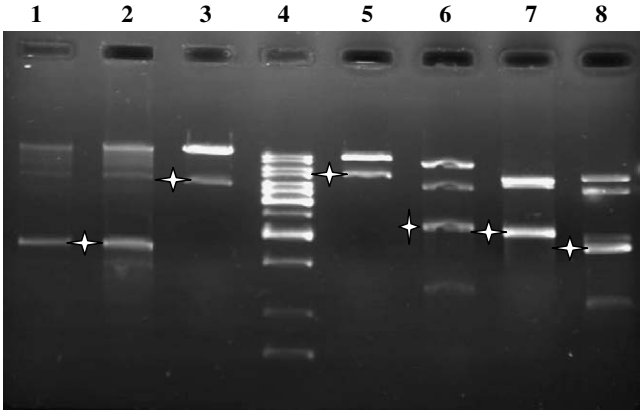

Fig. 3. Restriction digestion of pFBGGT. Lane 1, *Kpn*I and *Nhe*I (TK1, 1630 bp); lane 2, *Xho*I and *Age*I (TK 2, 1600 bp); lane 3, *Afl*II and *Age*I (NEO + DSHBG, 4050 bp); lane 4, Marker 1 kb(Fermentas); lane 5, *Afl*II and *Xho*I (NEO + DSHBG + TK2, 5650 bp); lane 6, *Kpn*I and *Mlu*I (USHBG, 2200 bp); lane 7, *Sac*I and *Afl*II (HBG, 2100 bp); and lane 8, *Sac*I and *Bgl*II (HYGRO, 1800 bp), The desired bands are signed by star.

Results of transfection cos-7 with naked DNA

Treatment of the culture with hygromycin and geneticin for 7 days resulted in survival of only 20 colonies to which ganciclovir was added and treatment continued for another 7 days leading to the survival of only 3 colonies (Fig. 4). These colonies were transferred to new culture vessels. DNA of these clones was extracted and analyzed by PCR. The results of PCRs were negative for TK1 and TK2 and positive for hygromycin, neomycin, and the fragment which included 300 bp above the USHBG in the genome, USHBG, and 400 bp of 5′ end of hygromycin expression cassette (Fig. 5). This latter PCR product was sequenced and the results of sequencing confirmed the homologous replacement of the construct in genome.

Discussion

Different strategies are available for gene therapy of β-thalassemia. Some of the frequently used strategies include: Application of viral vectors [4–7], gene targeting vectors [13,18,20], small fragment homologous recombination [21], triplex forming oligonucleotide [22], antisense snRNA [23], RNAi, and ribozyme [24]. The aim of this study was to make a novel double positive double negative construct for β-globin gene targeting. Gene targeting is used to knock in, knock out or introduce subtle mutations into a gene [25]. It is an ideal method for correcting genetic defects, because it does not affect other regions of the genome, so it does not affect tempo-spatial pattern or normal level of gene expression during the differentiation steps of the cells [26]. Up to now two kinds of gene targeting vectors have been exploited by researcher: replacement vectors and Insertion vectors. Replacement vectors are ideal for generating subtle mutations [27]. The construct described here is a replacement vector and primarily designed to correct subtle mutations of beta globin gene. According to positive–negative selection strategy (PNS) which was originally suggested by Mansour et al. [28] a gene targeting vector usually has the following elements: two homologous stems, positive and negative selection markers, and the desired target gene in the middle of this cassette. We chose upstream and downstream regions of haemoglobin gene as the homologous stems; neomycin and hygromycin resistance genes were the positive selection markers. Thymidine kinase gene was introduced at each end of the targeting cassette as negative selection marker to ensure selection of homologous recombinant cells versus randomly integrated ones. To make sure a stably transfected cell with this construct has received both homologue stems and the complete HBG gene, the cells can be positively selected by addition of hygromycin and geneticin to the medium. If the recombination happens between either of stems (USHBG or DSHBG) and the body of haemoglobin gene (Figs. 6B and C), instead of the other stem (Fig. 6A), the recombinant cell will contain only one of positive selection

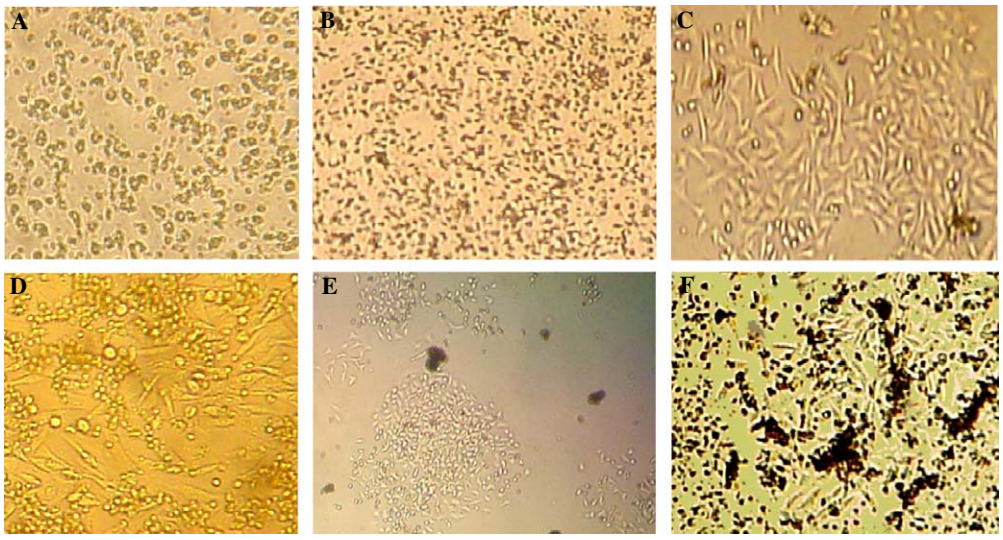

Fig. 4. Transfection of Cos-7 cells with naked DNA; untransfected cells (control) which were treated with hygromycin (A), G418 (B), and ganciclovir (C), in the case of (A,B). All of the cells were dead but abut ganciclovir the cells were alive, transfected cells which were treated with G418 and hygromycin after three days (D), after one week (E), A colony of cells after 2 week treatment with G418, hygromycin, and ganciclovir (F).

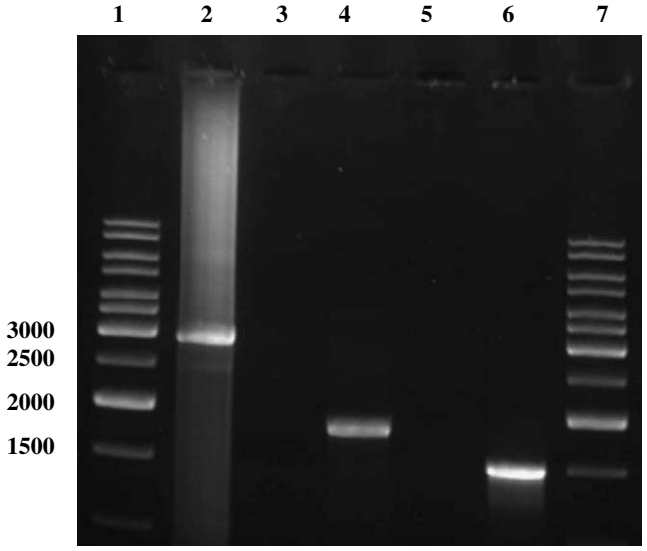

Fig. 5. Lane 1, PCR on DNA samples of selected cells; lane 2, (2985 bp contains a segment above of US + US + a segment of 5′ end of HYG); lane 3, (TK1 which is not amplified); lane 4, (hygromycin 1800 bp); lane 5, (TK2); and lane 6, (neomycin 1490 bp).

marker, so these cells will not survive in medium containing hygromycin and G418. On the other hand, if the targeting construct is randomly integrated within the genome, the termini of the targeting cassette will not drop and they will contain both or at least one copy of the thymidine kinase gene. Such cells will die when ganciclovir is added. In practice, however, random integrants are still recovered because of the damage to the negative selection marker during transfection, but putting two negative selection markers at both ends of the construct minimizes this possibility [29]. Survival after these positive and negative selection steps is a strong evidence for homologous recombination.

The optimum length of a homologous stem is between 2 and 10 kb [30]. Due to limitation in capacity of pBGGT, we chose relatively short 2.2 kb USHBG and 2.5 kb DSHBG fragments as the stems. All previously reported gene replacement constructs have benefited from just one positive selection marker between the two homologous stems [13,18,20,25,26]. To our knowledge, this is the first report of using a double positive selection strategy to ensure complete replacement of the desired target gene in one step of HR and with using one construct. In previous strategies, such as Hit and Run [31] or Tag and Exchange [26], the target gene is replaced during two steps of HR.

The HBG fragment amplified for this construct consists of a complete expression cassette of the gene: its original promoter, exons, introns, terminator, and 3′ enhancer which lies downstream of HBG [32]. Instead of direct cloning of the amplified fragments in pBGGT, we first cloned each fragment in pTZ57T/A cloning vector. These extra cloning steps provide the opportunity to sequence each fragment separately and choose the most suitable copy of the cloned gene to include it in the final construct. The host plasmid of the gene targeting cassette, pBGGT, had been basically designed to be used for this purpose, so all subcloning steps were performed directionally. This process saves time and cost.

The most problematic disadvantage of gene targeting strategy is the very low frequency of homologous recombination in mammalian cells. This rate is estimated to be only 10^{-5} to 10^{-7}[18]. Some approaches have been employed to improve the efficiency of HR such as, transient overproduction of an active recombinase like RecA or RAD51 which could increase the HR rate [33,34]. Recently, sequence-specific zinc finger nucleases have been used to induce double strand breaks in desired sites [35]. This sounds very promising for promoting gene targeting

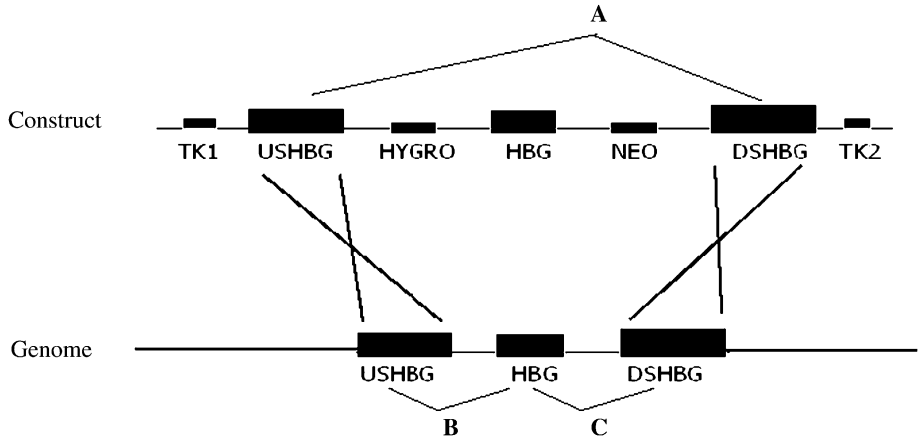

Fig. 6. The possibilities of crossing over: if recombination occurs between USHBG and HBG (B) or DSHBG and HBG (C), the resulting cells will die with double positive selection, but cells which undergo recombination between USHBG and DSHBG contain two positive selection markers and would be alive (A).

studies, since artificial double strand breaks at the ends of homologous regions in the genome and the construct can increase the HR rate [30,36]. Any progress in gene delivery systems also helps enhancing the chance of retrieving more recombinants in a cell population. The construct presented here is designed to ultimately introduce functional beta globin genes into haematopoietic stem cells of β-thalassemia patients. The sensitivity and little number of these cells are also other challenges to be overcome. This construct might be further modified by replacing the sequences of specific recombinase sites at the ends of positive selection markers to remove of these segments after selection steps [37]. However, even in its current format, it could be a promising tool to introduce normal genes to β-thalassemia HSCs.

References

[1] D.J. Weatherall, O.R. Gibbons Higgs, N.F. Olivier, W.G. Wood, The Thalassemia Syndromes, fourth ed., Black well science ltd, Italy, 2001, pp. (287-296) pp. (150-164) pp. (206-238).
[2] D. Rund, E. Rachmilewitz, New trends in the treatment of β-thalassemia, Oncol. Hematol. 33 (2000) 105–118.
[3] M. Sadelain, S. Rivella, L. Lisowski, S. Samakoglu, I. Riviere, Globin gene transfer for treatment of the β-thalassemias and sickle cell disease, Best Pract. Res. Clin. Hematol. 17 (3) (2004) 517–534.
[4] S. Imren, E.M. Fabry, A.K. Westernman, R. Pawliuk, P. Tang, M.P. Rosten, L.R. Nagel, P. Leboulch, J.C. Eaves, R.K. Humphries, High-level β-globin expression and preferred intragenic integration after lentiviral transduction of human cord blood stem cells, Clin. Invest. 114 (7) (2004) 953–962.
[5] E.F. Nicolini, S. Imren, II-H. Oh, K.R. Humphries, P. Leboulch, E.M. Fabry, L.R. Nagel, J.C. Eaves, Expression of a human β-globin transgene in erythroid cells derived from retrovirally transduced transplantable human fetal liver and cord blood cells, Blood 100 (2002) 1257–1264.
[6] C. May, S. Rivella, A. chadbum, M. Sadelain, Successful treatment of murine β-thalassemia intermedia by transfer of the human β-globin gene, Blood 99 (2002) 1902–1908.
[7] P. Malik, P.I. Arumugam, J.K. Yee, G. Puthenveetil, Successful correction of the human cooley's anemia (beta) thalassemia major phenotype using a lentiviral vector flanked by the chicken hypersensitive site 4 chromatin insulator, Ann. N.Y. Acad. Sci. 1054 (2005) 238–249.
[8] S.N. Templeton, D.D. Lasic, Gene and Cell Therapy: Therapeutic Mechanism and Strategies, second ed., Marcel Dekker, New York, 2003.
[9] Q. Li, W.D. Emery, M. Fernandez, H. Han, G. Stematoyannopoulos, Development of viral vectors for gene therapy of β-chain hemoglobinopathies: optimization of a δ-globin gene expression cassette, Blood 93 (1999) 2208–2216.
[10] I. Plavec, T. Papayannopoulou, C. Maury, F. Meyer, A human β-globin gene fused to the human β-globin locus control region is expressed at high levels in erythroid cells of mice engrafted with retrovirus-transducer hematopoietic stem cells, Blood 81 (1993) 1384–1392.
[11] S. Rivella, M. Sadelain, Genetic treatment of severe hemoglobinopathies: the combat against transgene variegation and transgene silencing, Semin. Hematol. 35 (1998) 112–125.
[12] Z.H. Li, P.D. Liu, X.W. Yin, C.Z. Guo, C.C. Liagn, Targeted correction of the point mutations of β-thalassemia and targeted mutagenesis of the nucleotide associated with HPFH by RNA/DNA oligonucleotides: potential for β-thalassemia gene therapy, Blood Cells Mol. Dis. 27 (2001) 530–538.
[13] H.Z. Lu, T.J. Books, M.R. Kaufman, J.T. Ley, Long targeting arms does not increase the efficiency of homologous recombination in the β-globin locus of murine embryonic stem cells, Blood 102 (2003) 1531–1533.
[14] H.S. Hahm, Y. Yi, K.D. Lee, J.M. Noh, L. Yun, S. Hwang, H.K. Lee, Construction of retroviral vectors with enhanced efficiency of transgene expression, Virol. Methods 121 (2004) 127–136.
[15] W. Walther, U. Stein, Viral vectors for gene transfer, Drugs 60 (2000) 249–271.
[16] S. Imren, E. Payen, A.K. Westerman, R. Pawliuk, E.M. Fabry, Permanent and panerythroid correction of murine β-thalassemia by multiple lentiviral integration in hematopoietic stem cells, PNAS 99 (2002) 14380–14385.
[17] S.B. Primrose, R.M. Twyman, R.W. Old, Principles of Gene Manipulation, sixth ed., Black well science Ltd, London, 2001.
[18] M.K. Vasquez, K. Marburger, Z. Intody, H.J. Wilson, Manipulating the mammalian genome by homologous recombination, PNAS 98 (2001) 8403–8410.
[19] J. Sambrook, D. Russell, Molecular Cloning, Third ed., CSHL Press, New York, 2001.
[20] O. Smithies, R.G. Gregg, S.S. Boggs, M.A. Koralewski, R.S. Kucherlapati, Insertion of DNA sequences into the human chromosomal β-globin locus by homologous recombination, Nature 317 (1985) 230–234.
[21] C.D. Gruenert, B. Emanuela, G. Novelli, A. Colosimo, B. Dallapiccola, F. Sangiuolo, K.K. Goncz, Sequence-specific modification of genomic DNA by small DNA fragments, Clin. Invest. 112 (2003) 637–641.

[22] M.M. Seidman, P.M. Glazer, The potential for gene repair via triple helix formation, Clin. Invest. 112 (2003) 487–494.
[23] M.M. Vacek, H. Ma, F. Gemignani, G. Lacerra, T. Kafri, R. Kole, High-level expression of hemoglobin A in human thalassemic erythroid progenitor cells following lentiviralvector delivery of an antisense snRNA, Blood 101 (2003) 104–111.
[24] N. Lan, P.R. Howrey, S.W. Lee, A.C. Smith, A.B. Sullenger, Ribozyme-mediated repair of sickle β-Globin mRNAs in erythrocyte precursors, Science 280 (1998) 1593–1596.
[25] A.D. Sorrell, F.A. Kolb, Targeted modification of mammalian genomes, Biotechnol. Adv. 23 (2005) 431–469.
[26] U. Muller, Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis, Mech. Dev. 82 (1999) 3–21.
[27] L.A. Joyner, Gene Targeting: A Practical Approach, second ed., Oxford University press, New York, 2000.
[28] S.L. Mansour, K.R. Thomas, M.R. Capecchi, Disruption of the proto-oncogene *int-2* in mouse embryo-derived stem cells :a general strategy for targeting mutations to non-selectable genes, Nature (1988) 336–348.
[29] J.M. Sedivy, A. Dutriaux, Gene targeting and somatic cell genetics-a rebirth or a coming of age? Trends Genet. 15 (1999) 887–890.
[30] A.V. Lanzov, Minireview gene targeting for gene therapy: prospects, Mol. Genet. Metab. 68 (1999) 276–282.
[31] V. Valancius, O. Smithies, Testing an ''in-out'' targeting procedure for making subtle genomic modifications in mouse embryonic stem cells, Mol. Cell. Biol. 11 (1991) 1402–1408.
[32] M. Trudle, F. Costantini, A 3′ enhancer contributes to the stage-specific expression of the human beta-globin gene, Gene Dev. 1 (1987) 954–961.
[33] G.O. Shcherbakova, A.V. Lanzov, H. Ogawa, V.M. Filatov, Over expression of bacterial RecA protein stimulates homologous recombination in somatic mammalian cells, Mutat. Res. 459 (2000) 65–71.
[34] A.A. Volodin, O.N. Voloshin, R.D. Camerini-Otero, Homologous recombination and RecA protein: towards a new generation of tools for genome manipulations, Trends Biotechnol. 23 (2005) 97–102.
[35] M. Mani, K. Kandavelou, J.F. Dy, S. Duria, S. Chandrasegaran, Design, engineering, and characterization of zinc finger nucleases, BBRC 335 (2005) 447–457.
[36] A. Dudas, M. Chovanec, DNA double strand break repair by homologous recombination, Mutat. Res. 566 (2004) 131–167.
[37] C.S. Branda, S.M. Dymecki, Talking about a revolution: the impact of site-specific recombinases on genetic analyses in mice, Dev. Cell 6 (2004) 7–28.