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Site-specific transfer of an intact β-globin gene cluster through a new targeting vector

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Abstract

The ideal gene-therapy vector for treating genetic disorders should deliver intact therapeutic genes and their essential regulatory elements into the specific "safe genomic site" and realize long-term, self-regulatory expression. For β-thalassemia gene therapy, viral vectors have been broadly used, but the accompanying insertional mutation and immunogenicity remain problematic. Hence, we aimed to develop new non-viral vectors that are efficient and safe in treating diseases. As previous studies have demonstrated that physiological expression of β-globin genes requires both a 5′ locus control region and 3′ specific elements, we constructed a new human chromosome-derived targeting vector to transfer the intact β-globin gene cluster into K562 cells. The whole β-globin gene cluster was precisely integrated into the target site and expressed in a self-regulatory pattern. The results proved that the human chromosome-derived vector was specifically targeted to the human genome and this could provide a novel platform for further gene therapy research.

Keywords: Site-specific integration; β-Thalassemia; Homologous recombination; Human β-globin gene cluster; Human chromosome-derived vector

The major goal of gene therapy is to treat genetic disorders by delivering a therapeutic gene and its associated regulatory elements into the nucleus of the host cells, in order to express the deficient gene products physiologically, without disrupting the normal gene expression and critical signaling pathways of the host cell [1].

β-Thalassemia is a group of hereditary disorders characterized by certain genetic deficiencies in the synthesis of β-globin chains. Generally, it can be caused by more than 200 kinds of mutations that lead to the accumulation of unmatched α-globin chains in matured red blood cells [2]. To cure this group of monogenetic diseases, the exogenous β-globin gene has to be stably integrated and appropriately expressed [3]. Previously, transfer of β-like globin genes mediated by viral vectors has been broadly used for β-thalassemia therapy. Their implementation, however, has been hampered by the difficulties in controllable expression of globin genes in the progeny of virally transduced cells and by the dangers of insertional mutation, production of wild virus, as well as potential immune response [4]. For example, the oncoretrovirus mediated globin gene transfer in targeted cells permits random integration of single copy transgene into the genome and erythroid-specific expression of the human β-like globin genes, but the expression level is low and affected by the chromosomal position effects [5]. In order to overcome the chromosomal position effects, a large locus control region (LCR) fragment was included in the transfer of β-globin genes by lentiviral vectors [6,7].

However, the safety issues still make the viral vectors problematic, especially after three children were reported to have developed T cell leukemia in a clinical trial for

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treatment of X-linked severe combined immunodeficiency (SCID) [8,9].

Non-viral vectors have important safety advantages over viral vectors, including their reduced pathogenicity and capacity for insertional mutation, as well as low cost and ease of production. In addition, recent transgenic mouse experiments demonstrated that an interaction between the γ-globin gene promoter and the LCR is required for γ gene activation [10], and full expression by LCR requires all five HSs and other specific elements, including 5′ promoter and 3′ enhancer [11,12]. Therefore, we need a new non-viral construct including the intact β-globin gene cluster for studying the treatment of β-thalassemia.

Because the whole β-globin gene cluster is too large for conventional non-viral vectors, previous studies have focused mainly on the transfer of functional genes plus miniLCR or HS4, HS3, and HS2 [6,7,13]. However, the smaller recombinant transgenes transferred were integrated randomly, often with accompanying genomic alterations or abnormal expression of the transgenes due to the integration sites and/or copy numbers [14,15]. Therefore, we have constructed a new vector—a recombinant bacterial artificial chromosome (BAC)-based targeting vector including two homologous arms derived from human chr7q36.1 and intact human β-globin gene cluster. Site-specific integration of the genes of interest may circumvent insertional mutation caused by random integration and the transfer of intact human β-globin gene cluster may favor of therapeutic gene expression in a self-regulatory manner.

Materials and methods

Construction and identification of the targeting vector. The targeting vector, BACMS is constructed from pBeloBAC11 vector by modifying homologous arms and positive/negative selection marker genes. In brief, two homologous arms are derived from *Homo sapiens* genomic clone MC169 (GenBank No. AF155875, submitted on 18-May-1999, National Laboratory of Medical Genetics of China) containing a 3.1 kb *Stu*I (75586)-*Kpn*I (78703) fragment. The pBeloBAC11 vector and the *Homo sapiens* genomic clone MC169 were digested by *Not*I and *Stu*I–*Kpn*I (78703), respectively, and then were blunted with T4 DNA polymerase. The recovered 3.1 kb fragment was ligated to the linearized pBeloBAC11. A neomycin resistance sequence (neo) driven by human cytomegalovirus immediate early promoter was inserted into the *Not*I–*Nhe*I sites between the homologous arms. The negative selection marker gene *HSV-tk* driven by mouse phosphoglycerate kinase-1 gene promoter, was inserted out of the 3′ homologous arm (see Supplementary material, S-Figure 1). The *Not*I (1618) and *Nhe*I (3113) sites were used for inserting the genes of interest, and the *Sgr*AI sites (11354, 12511) are used for linearization of recombination BAC before transfected.

Isolation of BACs (BAC186D7 and βD-BAC) containing the human β-globin gene has been previously reported [16,17]. The intact human β-globin gene cluster (97 kb) was derived from βD-BAC digested with *Not*I. The interest fragments were purified after separation by pulse field gel electrophoresis (PFGE) on 1% agarose gel (Roche Molecular Biochemicals), using the 5–150 kb auto-program of CHEF MAPPER (Bio-Rad), and then were ligated to linearized BACMS digested with *Not*I. The recombinant BACMS containing the human β-globin gene cluster (BACMS/BGGC) was confirmed by PFGE after digestion with *Mlu*I, *Xho*I, *Sal*I, and *Not*I, respectively.

Cell transfections and single cell isolation cloning. Human K562 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. Cells were seeded into each well of a 6-well plate 1 day prior to electroporation. For electroporation, the cells were resuspended at 2.5×10^6 cells/ml in warm growth medium. Electroporation of 1×10^6 cells with 10 μg of linearized BACMS/BGGC digested by *Sgr*AI in a 5 mm long chamber with a 100 mm^2 cross-section was carried out as described [18]. The electric pulse from a 950 μF capacitor charged to 250 V lasted for 30–35 ms. After electroporation, the cells were diluted in warm growth medium at a final concentration of approximately 1×10^4 cells/ml and plated at 100 μl per well in a 96-well plate. Seventy-two hours after electroporation, the medium was replaced by an equal volume of medium with 400 μg/ml G418. After about 10–15 days, the bulk of cells that were sensitive to G418 died. Resistant cell clones that were actively proliferating were picked up and transferred to a 24-well plate. After about 20 days, G418 resistant cell clones were transferred to T25 flasks to reach confluence. The cell clones were then grown in medium with gancyclovir (10 μg/ml).

Southern blot. After transfection and selection with the corresponding antibiotics, the transformant cells were harvested. The genomic DNA was extracted from the cells, separated by electrophoresis on 0.8% agarose gels after digestion by restriction enzymes, and transferred to nylon membranes (Hybond N^+, Amersham, USA). Blots were UV cross-linked and hybridized with [α-^{32}P]dCTP labeled probes. The probes used for hybridization with the β-globin gene cluster are listed below in the 5′–3′ direction with respect to their position in the β-globin region: HS2/*Bgl*II–*Hin*dIII (0.7 kb), RI3.3/*Eco*RI (3.3 kb), ^{G}γ-globin/*Sna*BI–*Bam*HI (1.7 kb), β-globin/*Eco*RI–*Bam*HI (0.9 kb), and RK29/*Eco*RI (1.2 kb). The copy numbers of the β-globin gene cluster were quantified using the PhosphorImager (Molecular Dynamic). The probe used to analyze the integration sites in the cell clones was the neo gene (0.4 kb).

G-banding analysis and fluorescent in situ hybridization (FISH). Preparation of chromosome spreads from K562 cells and chromosome analysis were carried out according to standard cytogenetic procedures. The better metaphases were noted at the coordinates. A gray level charge coupled device (CCD) Ikaros camera (Japan) was used. The chromosome spreads were treated as follows: decolorized in 100% methanol for 10 min at room temperature, air-dried, digested with 0.02% pepsin at 37 °C for 5 min, washed three times in PBS, fixed in 6% paraform for 5 min at room temperature, washed three times in PBS, dehydrated serially, and then air-dried. About 1 μg of purified BACMS/BGGC plasmid DNA was labeled with tetra-methyl-Rhodamine-6-dUTP (Roche, USA) by random primer labeling kit according to the manufacturer's suggestion (Roche, USA). The labeled probe was hybridized with metaphase chromosome spreading decolorized as previously described. Chromosome spreads were mounted and counterstained in Vectashield antifade containing DAPI. Hybridization signals in the noted metaphases were detected under an Axiolab HBO 50/ac epifluorescence microscope (Carl Zeiss, Oberkoken, Germany). Image acquisition and analysis of DNA-FISH were performed by attached software.

Real-time quantitative RT-PCR. Total RNA was isolated from cells using TRIzol (Invitrogen) and reverse-transcribed using the reverse transcription system (Promega) according to the manufacturer's protocol. PCR amplification of different genes was performed using EXTaq polymerase (Takara), with a program of 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 54–62 °C for 30 s, 72 °C for 30 s, and extension at 72 °C for 10 min. Quantitative RT-PCR analysis was performed on an ABI PRISM 7300 Sequence Detection System using the SYBR Green PCR Master Mix (Applied Biosystems). The PCR consisted of 12.5 μl SYBR Green PCR Master Mix, 0.8 μl of 10 mM of forward and reverse primers, 10.4 μl water, and 0.5 μl template cDNA in a total volume of 25 μl. Cycling was performed using the default conditions of the ABI 7300 SDS Software (Ver. 1.3.1): 2 min at 50 °C, 10 min at 95 °C, followed by 40 rounds of 15 s at 95 °C, and 1 min at 60 °C. The relative expression of each gene was normalized against β-actin. The primers for quantitative RT-PCR are shown as supplemental materials (S-table 2).

Results

Identification of recombinant targeting vectors (BACMS/BGGC)

Different fragments were obtained after digestion of recombinant targeting vectors BACMS/BGGC with *Not*I, *Mlu*I, *Xho*I, and *Sal*I, respectively (S-Table 1). It is shown in S-Figure 2 that there are corresponding fragments as expected after *Mlu*I, *Sal*I, *Xho*I, and *Not*I digestion, respectively. The human β-globin gene cluster was inserted into the targeting vector BACMS in reverse direction to *neo* transcription.

Determination of the integration sites and integrity

This recombinant targeting vector containing the entire human β-globin gene cluster was electroporated into K562 cells followed by positive selection using G418. Twelve G418 resistant cell clones were obtained. After the negative selection, two gancyclovir resistant K562 cell clones were isolated and designated IIG2 and IIG12, respectively.

Except three clones contaminated, nine cell clones were chosen for analysis by Southern blot (Fig. 1). The results showed that a 7.8 kb band was generated by *Bgl*II digestion of genomic DNA from IIG2 to IIG12, respectively; and that a 7.8 kb band and a 5.0 kb band were generated by *Bgl*II digestion of genomic DNA from the IC1 cell clone (Fig. 1). The results indicate that targeted recombination may occur in IIG2 and IIG12 cell clones, and that the IC1 cell clone may include both targeted and random integration.

To investigate the integration sites of transgenes, we performed G-banding and DNA-FISH analysis in the cell clones. Hybridization signals at 11p15 and 7q36.1 were observed on chromosomes from cell lines IIG2, IIG12, and IC1 (Fig. 2). Additional hybridization signal at 1qter was observed in cell line IC1. The signal at 11p15 is consistent with hybridization at the endogenous globin locus while the signals at the other two loci are possibly derived from non-specific random integration events. The transfection efficiency and targeted integration efficiency were 1.2×10^{-5} ($12/1 \times 10^{6}$) and 16.7% (2/12), respectively.

Southern blot analysis was also used to further investigate the integrity of the foreign gene cluster in positive cell clones using five probes labeled with [α-^{32}P]dCTP (Fig. 3A). For each *Eco*RI digested genomic DNA from these cell clones and K562 cells, there were five bands (including 10.5 kb HS432, 7.0 kb β-globin gene, 5.5 kb ^{A}γ-globin, 3.3 kb HS5, and 1.0 kb 3′HS1) generated (Fig. 3B). The copy number of these cell clones was estimated by comparing every band of genomic DNA to a known amount of plasmid DNA, which showed that the copy numbers of every composition of the cell clones are approximately 1–2 times higher than the reciprocal one in K562 cells (Fig. 3C). The above results indicate that the four transgenic cell lines each contain the entire LCR, 3′HS1, and functional β-like globin genes in one intact fragment.

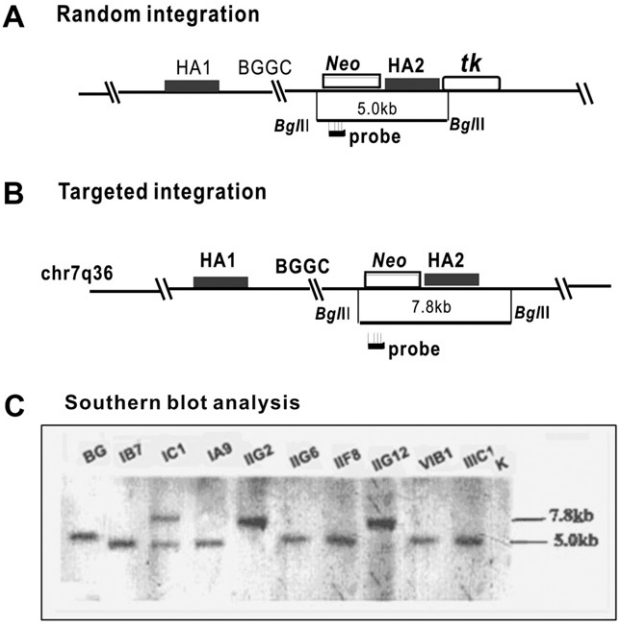

Fig. 1. Analysis of site-specific integration. There are two *Bgl*II sites in BACMS/BGGC vectors, one of which is located at the 3′ end of the β-globin gene cluster (97.3 kb), and the other is located at the 5′ end of the *HSV-tk* gene (102.4 kb). The *Bgl*II site in chr7q36.1 is located downstream at the 3′ end of HA2 (3.3 kb). (A) Random integration occurs between other chromosomes and targeting vectors. A 5.0 kb fragment could be obtained using neo gene probes. (B) Homologous recombination occurs between the targeting vector and chr7q36.1. The human β-globin gene cluster is inserted into the region of chr7q36.1. A 7.8 kb fragment could be obtained using neo gene probes. (C) Southern blot analysis of site-specific integration. The probe used in hybridization is neo labeled with [α-^{32}P]dCTP. Lane BG, BACMS-BGGC plasmid/*Bgl*II; lane IB7 through K, genomic DNA from positive cell clones and K562 cells digested by *Bgl*II.

Quantitative detection of ^{A}γ-globin gene expression level in the integrated clones

The transcription levels of the β-like globin genes in positive cell clones were measured using a quantitative RT-PCR. We detected only the expression of ^{A}γ-globin gene in these cell clones because of fetal erythrocytes properties [19]. Since there are two copies of ^{A}γ-globin genes in a normal K562 cell line, the relative expression of each gene was normalized against β-actin. K562 cells and IIG2, IIG12, IC1, and IB7 clones were treated with hemin for 48 h; more than 90% of the cells underwent differentiation, which was confirmed by 3,3′-diaminobenzidine dye (data not shown). Following three times quantitative RT-PCR for each sample, the mean values of ^{A}γ-globin mRNA were calculated. The expression level of human ^{A}γ-globin gene was higher 1–4 times in IIG2, IIG12, IB7, and IC1 cell clones than that of control (Fig. 4), which indicated the self-regulatory manner of ^{A}γ-globin gene expression in the integrated clones.

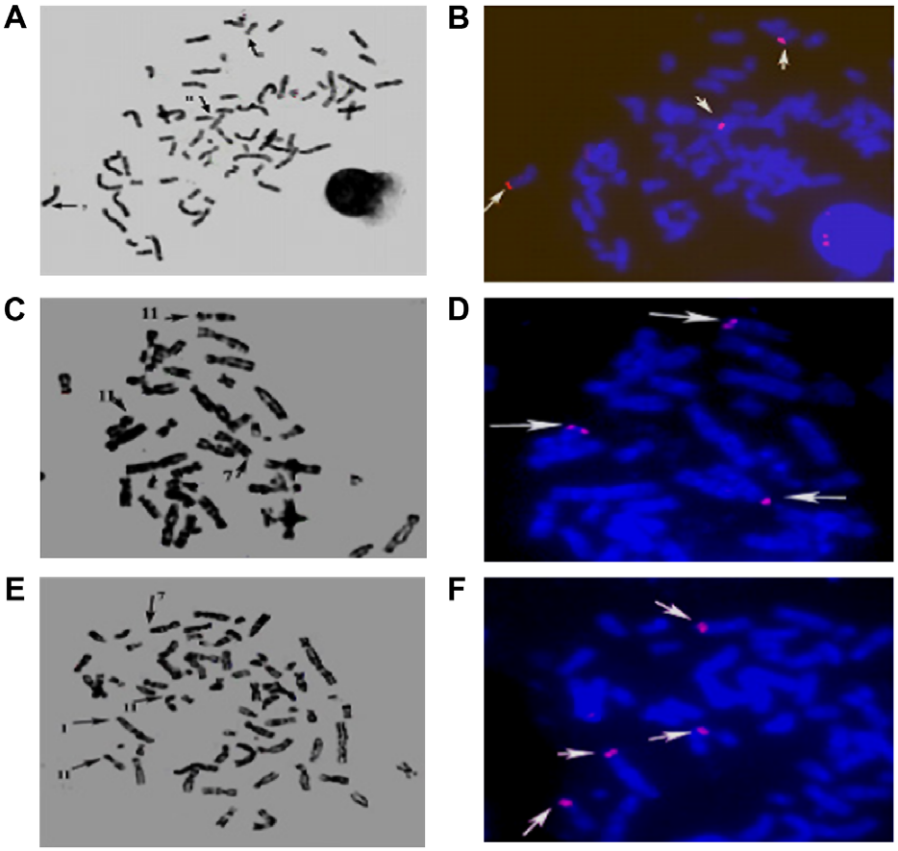

Fig. 2. G-banding and DNA-FISH analysis of integration sites. (A,C,E) G-banding analysis of IIG2, IIG12, and IC1 cell clones, respectively; (B,D,F) FISH results of the same metaphase corresponding to Fig. 3A, C, and E, respectively. Arrows indicate where the signals appeared on the chromosomes. Three signals were observed on chr11pter and chr7qter in the IIG2 and IIG12 cell clones. Four signals were observed on chr11pter, chr1qter, and chr7qter. The probes used in the hybridization were BACMS/BGGC plasmid labeled with tetra-methyl-Rhodamine-6-dUTP.

Discussion

Our results demonstrate that the targeting vector, derived from human chromosome, is efficient for site-specific integration of large exogenous genes into mammalian genomes. Unlike previous oncoretroviral vectors, transfection of K562 by this BAC-based vector containing β-globin gene cluster led to stable and self-regulatory transgene expression that was maintained throughout prolonged cultivation.

Indeed, viral vector-mediated transfer in mouse haematopoietic stem cells permits erythroid-specific expression of human β-globin gene, but the expression level is low and limited by chromosomal position effects [20,21]. Entire β-globin gene cluster transfer mediated by BAC or YAC vector has only been carried out in transgenic mouse [10]. In addition, the strategy of site-specific integration was adopted in order to reduce insertional mutations. Even though studies of site-specific recombinases, e.g., Cre, Flp, and φC31 integrase, which recognize certain DNA sequences in the genome and catalyze the recombination [4,22,23], have increased recently due to their higher frequency, we still prefer HR mediated integration, which has higher specificity.

To find a relatively safe region in human genome for exogenous genes' integration, we analyzed the MC169 clone in whole human genome using UCSC Genome Browser (http://genome.ucsc.edu). Sequence analysis indicated that the fragments from 75,586 to 78,703 in this clone share 96.1% identity with the region at chr7q36.1. There is only one gene—myeloid/lymphoid or mixed-lineage leukemia 3 (MLL3) located at this region (S-Figure 3); therefore we select the 3.1 kb fragment in this clone to act as the homologous arms in the targeting vector. Although integration at the defined location on the chromosome through HR, especially with a very large insertion fragment, is an extremely rare event, we obtained two targeted integration cell clones through the revised positive plus negative selection. Targeted integration of the intact β-globin gene cluster at chr7q36.1 was then proved by Southern blot and DNA-FISH analysis. LCR, located 6–20 kb upstream of the β-globin gene and another developmentally stable DNase I HS (3′HS1) site, located in regions downstream from the β-globin gene, are generally acknowledged to be necessary for the high level expression of globin genes in erythroid tissues [24–27]. Copy number analysis indicated that integration fragments are 1–2 copies higher in the

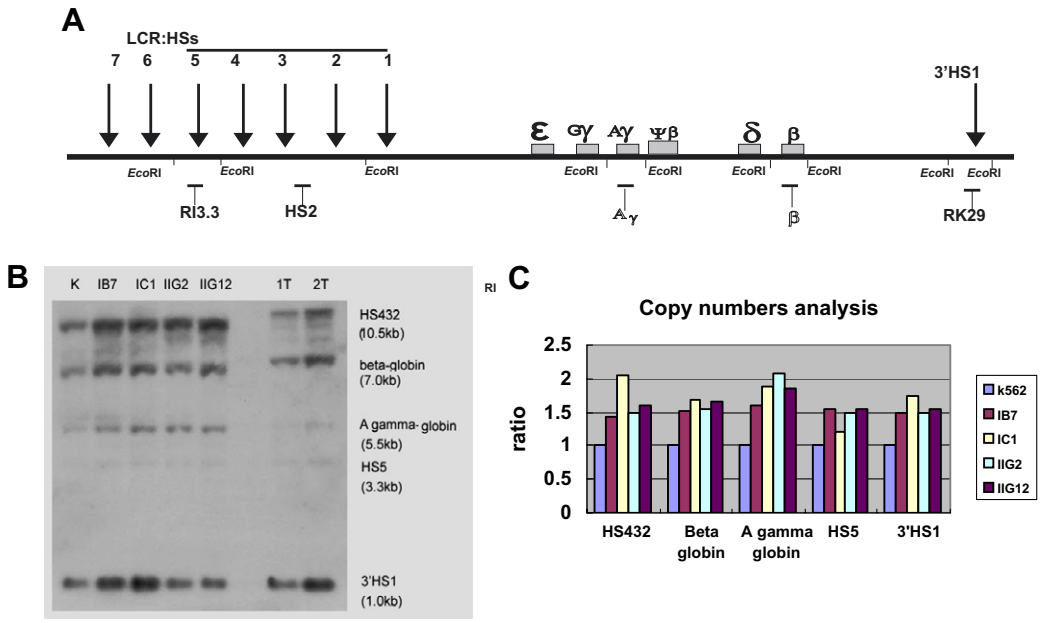

Fig. 3. Copy numbers analysis of the foreign gene cluster by Southern blot. (A) Physical map of the human β-globin cluster (97 kb). The solid squares and the arrows indicate the positions of the genes and the HSs, respectively. Parts of the restriction sites of *Eco*RI are shown. The closed boxes represent the positions of the probes used to map the human β-globin gene cluster in Southern blot analysis. (B) Southern blot analysis of *Eco*RI-digested DNA prepared from positive cell clones and K562 cells. The probes used in hybridization were HS432, β-globin gene, $^{A}\gamma$-globin gene, HS5 and 3′HS1. Lane IB7, IC1, IIG2, and IIG12, positive cell clones; lanes 1T and 2T, one copy and two copies of BACMS/BGGC; lane K, K562 cells. (C) Copy number analysis of positive cell clones. Each composition of the β-globin gene cluster of positive cell clones is 1–2 times that of normal K562 cells.

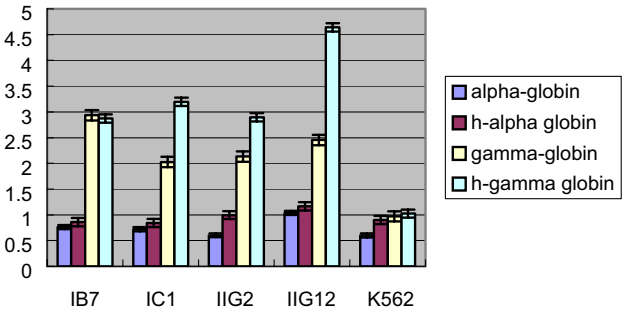

Fig. 4. Expression analysis of the human $^{A}\gamma$-globin gene by real-time RT-PCR. The h-alpha globin, h-gamma globin indicated that total RNA was isolated from IC1, IB7, IIG2, IIG12 cell clones and K562 cells after hemin induction.

positive cell clones than those of the control. Four clones were preferentially selected to detect expression level of β-like globin genes, including IC1 cell clone, IB7 cell clone, IIG2, and IIG12 cell clones. Because β-like globin genes express in copy number-dependent manner, the results of quantitative RT-PCR showed that the expression level of $^{A}\gamma$-globin gene in the positive cell clones is higher 1–4 times than that of normal K562 cells indicating self-regulatory expression.

Additionally, the correct function of the LCR may require a stepwise activation that starts in the transcriptional environment of an uncommitted cell and it is completed when the locus finds itself in the transcriptional environment of the cells of the erythroid lineage [28]. The K562 cell line has the properties of fetal erythrocytes and mainly expresses the $^{A}\gamma$ globin gene of human β-like genes after hemin induction [29]. Our results and previous reports implies that the site-specific transfer of the intact β-globin gene cluster through the new vector can also be used in other adult erythroid cell lines (e.g., KEMO and F-36P cell lines) or stem cells to realize self-regulatory gene expression. The principles underlying our cell model provide a paradigm for other gene clusters requiring stable and regulated expression of the site-specific transgenes or for investigating regulation of gene expression at the cellular level.

The results indicate that the new targeting vector can achieve efficient, long-term gene expression. With precise homologous arms, transgenes can be site-specifically integrated into the mammalian genomes, which might improve the safety of therapeutic usage. Once the problems relating to the integration sites at human chromosomes and transfection or integration efficiency concerns are fully addressed, the targeting vector containing the entire human β-globin gene cluster will be a high-efficiency tool for in vitro and in vivo β-thalassemia gene therapy, as well as for studying the regulation of β-globin gene expression.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.074.

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