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PRECISE AND SITE-SPECIFIC EDITING OF THE CHO HOST GENOME VIA CRISPR/CAS9 FOLLOWED BY THE INTEGRASE SYSTEM TO GENERATE A RECOMBINANT ARYLSULFATASE B PRODUCING CELL LINE

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Abstract
Stable recombinant cell line development by site direct integration of a gene of interest is critical issue in therapeutic proteins production such as arylsulfatase B enzyme. Deficiency of this enzyme in human body causes mucopolysaccharidosis type VI diseases which is treated by recombinant arylsulfatase B enzyme replacement. So in this work combination of homologous recombination (HR) method through clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 and integrase system were employed to precise integration of ARSB gene into the Chinese hamster ovary (CHO) host genome for stable production of recombinant enzyme. CHO-k1 cells were cultured and transfected by green fluorescent protein (GFP) donor plasmid and pX330 vector that targeted Rosa26 locus in the host genome by CRISPR/Cas9 technique. Then GFP positive cells were selected and edited through cassette exchange integrase system to convert recombinant arylsulfatase B producer cell line. Analysis of recombinant cell lines by ELISA verified arylsulfatase B enzyme expression in all GFP negative single clones after several passages. In conclusion using a precise and specific site direct integration method for genetic manipulation can lead to stability and continuous production of recombinant protein in modified cell lines.

Keywords: Recombinant cell line development, arylsulfatase B enzyme, CRISPR/Cas9, GFP.

Introduction

Recombinant therapeutic proteins are major part of biopharmaceutical drugs that have reached the US and EU market in recent decade (1). Many of recombinant therapeutic proteins are produced in mammalian cells because of their ability to post-translational modifications that generated proper glycosylation profile in final product (2). These glycosylated proteins are similar to human natural proteins with high stability and half-life in blood circulation as well as low immunogenicity (3). Chinese hamster ovary (CHO) cell–based systems is the common mammalian expression platform for production of recombinant therapeutic proteins (1). CHO cells is able to perform correct post-

translational modifications for proper assembly and protein folding(4). However, they lose the ability to produce foreign protein during long-term culture (5).

In recombinant cell line development process, the random integration of a gene of interest (GOI) into the structurally instable CHO genome may be influenced by the epigenetic state of the integration site. This position effect is due to variable accessibility of integration sites for gene expression system that reduced production yield over time (4, 6). Therefore, generation a stable recombinant cell line with constant yield and quality during the production process is essential issue (5). Other approach for this propose is site-specific transgene integration into the known hot spot locus of gene expression on the host genome (7, 8). In a recent study to ensure the correct orientation and integration site of transgene in the host genome, two approaches have been utilized, homologous recombination by transcription activator-like effector nucleases (TALEN) and Integrase methods (9). One of the latest known methods for precise genome engineering is CRISPR/Cas9 (10, 11). Cas9 is a nuclease targeted by small guided RNAs representing a high specific, easy to design and efficient gene editing system for a variety of cell lines (12, 13).

Similarly, we used an accurate genome editing system including CRISPR/Cas9 and dual integrase to generate a recombinant cell line producing a therapeutic enzyme called arylsulfatase B. Arylsulfatase B or N-Acetylgalactosamine 4-Sulfatase belongs to the arylsulfatase enzymes group that found in the lysosomes of the liver, pancreas and kidneys in the human body. Main function of this enzyme is the hydrolysis of sulfates by breaking down glycosaminoglycans (GAGs), dermatan sulfate and chondroitin sulfate (14). Arylsulfatase B is encoded by the ARSB gene (NCBI Gene ID: 411) with more than 130 known mutations that can lead to the deficiency of this enzyme in the human body. Accumulation of glycosaminoglycans in the lysosomes due to lack of arylsulfatase B enzyme causes mucopolysaccharidosis type VI (MPS VI).

MPS VI or Maroteaux-Lamy syndrome is a rare genetic disorder in which the involvement of various body organs progresses even years after diagnosis in infancy and in its severe form leads to death in adolescence. An effective treatment for MPS VI is enzyme replacement therapy with galsulfase drug that can improve walking and the ability to climb stairs. Galsulfase is recombinant form of human N-Acetylgalactosamine 4-Sulfatase (rhARSB) with a molecular weight of approximately 56 KD. This single chain glycoprotein is comprised of 495 amino acids and is produced in a genetically engineered Chinese hamster ovary derived cell line (15).

In this work, we first targeted specific locus in the CHO genome by CRISPR/Cas9 method to introduce *attP* sites for two phage integrases, phiC31 and Bxb1 as well as gene of green fluorescent protein (GFP) as protein marker in the donor vector. Targeted specific locus is named Rosa26 which is one of the best known site for high transgene expression, without change in phenotype or viability of cells. The Rosa26 locus encodes a nuclear RNA that expressed in a broad variety of tissues in mice (16) that many transgenic cell lines were created by this locus (17, 18). In the next step, cassette exchange was performed by phiC31 and Bxb1 integrase with different specific recognition site. As a consequence, gene of interest ARSB was inserted into the Rosa26 locus in the CHO-k1 genome and created recombinant ARSB cell lines. Subsequent analysis showed stability of rhARSB cell lines.

Material and Methods

Vector construction

Donor vector was designed to include the following elements, homology arms of Rosa26 locus plus *attP* sites on both sides, EGFP gene and hygromycin B resistant gene in addition to other expression necessary sequences.

Cloning of the Rosa26 sgRNA (Forward:5′-CACCGCAAGCGTGAGCATAAAACTC-3′and Reverse:5′-AAACGAGTTTTATGCTCACGCTTGC-3′) in the pX330-U6-Chimeric-BB-CBh-

hSpCas9 (Addgene#42230) vector performed according to Zhang lab protocol. In this way, first pX330 plasmid digested by BbsI restriction enzyme, then forward and reverse guide sequence oligo strands was annealed, finally ligation reaction between annealed oligo duplex with digested pX330 vector was done. For the next step, exchange cassette vector consisted of phiC31 and Bxb1 *attB* sites at the same side of donor vector, the arylsulfatase B (ARSB) gene (NCBI Gene ID: 411), SUMf1 gene (NCBI Gene ID: 285362) and Zeocin resistant gene. All constructs optimized based on the CHO-K1 codon usage (Figure 1).

Cell culture

The CHO-K1 cells (ATCC-CCL-61) were cultured in HamF12 medium (Biowest, France) supplemented with 10% Fetal Bovine Serum (Biowest, France) with 5% C02 and 95% humidity, at the temperature of 37°C. Recombinant rhARSB cell lines were generated by genetic manipulation of CHO-k1.

Transfection and recombinant clone selection

For the first step of rhARSB cell line development process, CHO-k1 cells were cultured to reach 70 percent confluence. Then the cells were plated at the density of 0.2–0.3 $\times 10^6$ cells/mL in a 12-well plate (Nest, Germany) about 24 hours before the transfection. Transfection was performed by GFP donor vector and pX330 at the ratio of 2:1 (v/v) using lipofectamine 2000 reagent (Thermo Fisher, US). GFP positive cells appeared after 72 hours and selected by addition of hygromycin B (Merck, Germany) as selection marker to the cell culture medium at the concentration 100µg/ml. The selection pressure for 7 days was maintained. Then single clone cells were selected by dilution method. GFP positive single clone cells were expanded and freeze for next step of transfection.

In the continuation of the process, GFP positive single clone were cultured to transfect by ARSB vector, pCMV-Bx (Addgene#51552) and pCS-kI (Addgene#51553) integrase vectors by 2:1:1 (v/v) ratio. Transfected cells were exposed to Zeocin (Thermo fisher, US) at 500 and 600 µg/ml concentration for 5-7 days, after that, the surviving cells were washed and got ready for colony selection by dilution in 96 well plate. The recombinant rhARSB single colons were picked and expanded to investigate of ARSB expression after several passages.

Genomic integration

Integration of ARSB gene into the host cell genome was verified by specific Fa (5′-CCTGTTTCTGTACCTGGCTTTG-3′) and Ra (5′-GTTCTTGTCCTGGATGAAGTCG-3′) primers using Polymerase Chain Reaction (PCR). Genomic DNA of rhARSB single colon cells were isolated by DNA Extraction kit (Favorgen, Taiwan).

Protein expression

Sandwich ELISA was performed for ARSB expression verification using Elabscience Human ARSB ELISA kit as follows: samples and standards were added to pre-coat with specific antibody to Human ARSB micro plate wells. Afterwards biotinylated detection antibody specific for ARSB were added to each well and incubated. Then avidin horseradish peroxidase (HRP) conjugate were added. Free components were washed away. Finally, the substrate solution was added to each well and terminated by stop solution. The optical density (OD) was measured at 450 nm ± 2 nm wavelength. Concentration of ARSB in the samples was calculated by comparing the OD of the samples to the standard curve.

Figure1. Map of **a.** GFP donor vector and **b.** rhARSB vector

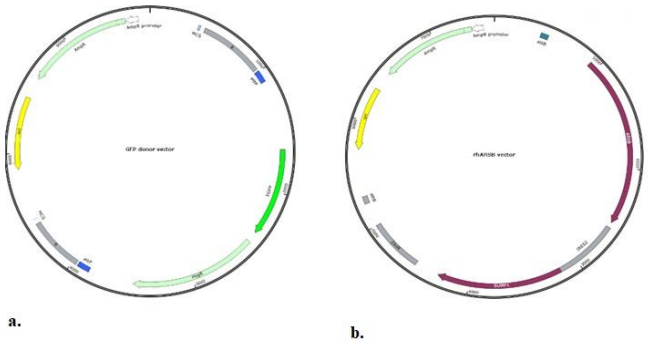


Figure 2. Recombinant cell pools that expressed GFP in cell culture were observed in green under inverted florescent microscope.

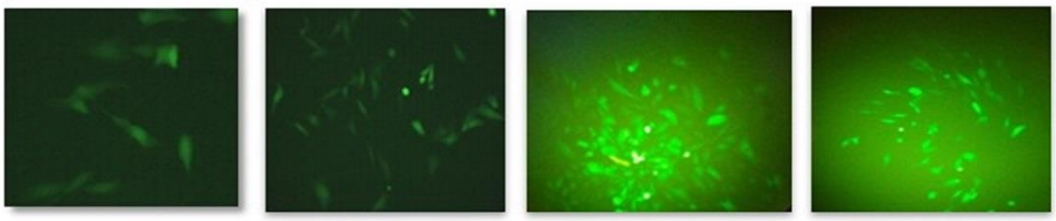
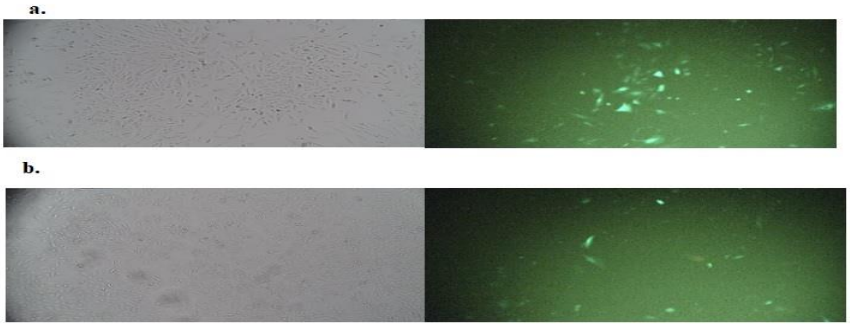


Figure 3. Expansion of GFP positive single cell colony generated a recombinant GFP cell lines.

Figure 4. Increasing selection pressure by **a.** 500 µg/ml and **b.** 600 µg/ml zeocin concentration led to higher GFP negative cells expansion which potentially considered as rhARSB recombinant cells.



Results

Green fluorescent positive cells by Homologous Recombination

Transfected cells by GFP donor vector created pool of recombinant cells. Analysis of this cell pools using inverted florescent microscope (Labomed TCM400) showed the presence of both positive and negative green florescent cells (Figure 2). These results indicated that homologous recombination by CRISPR/Cas9 in Rosa26 locus was performed successfully in the CHO-k1 host cell and GFP expressed in recombinant cells. For single cell colony selection only positive green florescent cells were picked that expressed GFP marker. Expansion of these cells led to the creation of GFP cell lines which were used for the next stages of recombinant cell line development (Figure 3).

Integration of ARSB gene through cassette exchange

For replacement of galsulfase gene with GFP gene in the Rosa26 locus, phiC31 and Bxb1 integrase were expressed transiently along with introducing ARSB vector. These integrases catalyze recombination between *attP* sites on the host genome of GFP positive cell line and *attB* sites beside the ARSB donor vector. We expected that green fluorescent positive cells would be converted to negative cells after precise ARSB gene replacement. Selection pressure through higher zeocin concentration indicated that the number of positive green cells was decreasing (Figure 4). After that, GFP negative cells were picked by dilution method and seven selected single colony cells named by A1, A2, A3, A4, A5, A6, and A7 were expanded to further analysis. PCR reaction was verified the cassette exchange and insertion of ARSB gene in the genomes belong to rhARSB recombinant cell lines.

Expression of arylsulfatase B enzyme

All of seven recombinant cell lines: A1, A2, A3, A4, A5, A6, A7 were cultured to investigate expression of rhARSB enzyme. Supernatants of cell culture were harvested after 5, 7 and 9 passages of cells for sandwich ELISA analysis. Concentration of enzyme production in every recombinant rhARSB cell lines calculated according to the standard curve that confirmed expression of arylsulfatase B enzyme in all cell lines (Table 1). These results showed that the modified cells were able to maintain the power of recombinant protein production after multiple passages and were stable.

Discussion

Site directed integration precise and efficiency are the important aim between the different genes of interest insertion methods. CRISPR/Cas9 system led to accurate integration of GFP donor vector into the Rosa26 locus directly without specific platform requirement such as prior insertion of recombination sites like to other recombinase systems. Rosa26 was selected as specific locus with minimum off target that generated GFP positive stable cell line for several passage.

Despite the effective capabilities of CRISPR/Cas9 method, one of the main problems of using this method is related to the low efficiency of large construct insertion. Combination of CRISPR/cas9 method with recombinase platform creation by insertion of *attP* sites that flanked GFP construct is able to overcome size limitation. The simultaneous use of dual site specific integrases, phiC31 and Bxb1 have given the power of specific and controlled *attP–attB* recombination.

In a similar previous study, Zhu and colleagues used DICE technique by combination of TALEN and double integrase system for genetic engineering of human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) in H11 locus (9). But in this work, we employed CRISPR/Cas9 method for genetic modification of CHO cell line with Rosa26 locus targeting and same double integrase recombination system for cassette exchange.

Also, site direct integration by this method makes possible precise genomic edition with a clean background for different multiple lines development in same location. Furthermore, addition of extra

GFP construct at the other location of host genome such as H11 locus as well as Rosa26 loci in the first generation of GFP positive cell lines can be increase gen of interest copy number.

At the other hand, Expression of GFP provides an efficient way for screening of recombinant cells through green florescent phenotype in the first step of cell line genetically modification and switch to negative GFP cells in the following step for create rhARSB cell line. However, screening through antibiotic resistance has also been applied at the same time by hygromycin B in first phase and zeocin in second phase of recombinant cell line development. In this study, we investigated the stability of the modified cell lines to continuous foreign protein production even after several generations and further, by optimizing the protein expression conditions, an efficient recombinant therapeutic drug production strategy can be achieved.

Conclusions

In conclusion, recombinant cell line development by precise editing method can guarantee site direct integration of transgene for steady product protein expression. Using CRISPR/Cas9 and then dual integrase system for genetic modification of CHO cell created a stable rhARSB cell line with continuous production ability.

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