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CELL LINE DEVELOPMENT THROUGH PIGGYBAC TRANSPOSON SYSTEM FOR THERAPEUTIC LYSOSOMAL ENZYMES PRODUCTION, ELOSULFASE ALFA AND IMIGLUCERASE, IN THE CHO CELL LINES.

Seyed Mehdi Hassanzadeh[1*], Azadeh Mirfeizollahi[2], Jafar Nikzad[3], Elahe Omidi[4], Hooman Kaghazian[5*]

[1,5*]Department of Research and Development, Research& Production Complex, Pasteur Institute of Iran, Tehran
[2]Department of Molecular Biology and Genetic Engineering, Farnad Fanavar Tasnim Pharmed Company, Tehran, Iran.
[3]Department of Drug Design, Pad tan Pharmed Imen Zist Company, Tehran, Iran.
[4]Department of Research & Development, Taranom Pharmed Co, Alborz, Iran

***Corresponding Author:** Hooman Kaghazian
*****Department of Research and Development, Research& Production Complex, Pasteur Institute of Iran, Tehran, Email: kaghazianh@pasteur.ac.ir, Tel: +989392055373; Fax: +982636102900

Abstract
lysosomal storage diseases are treated using recombinant enzyme replacement in Morquio A syndrome by Elosulfase alfa and Gaucher disease via Imiglucerase. Production of these recombinant enzymes with high quality need to appropriate genetic manipulation of the host genome. In this work, PiggyBac transposon system was designed for expression of elosulfase alfa and imiglucerase in the Chinese hamster ovary (CHO) cells. Three transposon vectors including PB::GALNS, PB::GBA, and PB::GFP were constructed separately and together with SuperPBase vector were transfected into the CHO-K1 cells. Then recombinant cell lines were selected by exposure to zeocin. The appearance of green fluorescent cells by PB: GFP/Super Base vectors showed the efficiency of the designed transposon system. The expression of elosulfase alfa in the GALNS recombinant cell lines and imiglucerase in GBA recombinant cells were confirmed by ELISA. In conclusion, The PiggyBac transposon system can be considered as an efficient tool to modification the CHO host genome for the development of recombinant cell lines to produce therapeutic enzymes.

Key Words: Transposon, cell line development, Elosulfase alfa, Imiglucerase, PiggyBac

Introduction
Enzyme replacement therapy is an efficient treatment in lysosomal storage diseases such as Morquio A syndrome and Gaucher disease. These chronic, rare inherited diseases was caused by accumulation of mucopolysaccharides in the body and damages the cells that developed by malfunction or lack of native enzymes inside the lysosomes (1). Morquio A syndrome is caused by the deficiency of N-acetylgalactosamine-6-sulfatase (GALNS) that lead to accumulation of excessive glycosaminoglycans (GAGs), keratan sulfate and chondroitin-6-sulfate in the lysosomes. A recombinant form of the human lysosomal enzyme N-acetylgalactosamine-6-sulfatase (GALNS)

called Elosulfase alfa was approved by the FDA in 2014 for treatment of Mucopolysaccharidosis type IVA or Morquio A syndrome (2).
Imiglucerase is an another recombinant enzyme used in the treatment of Gaucher disease was approved in 1994 by the FDA (3). Gaucher disease is the result of glucocerebrosidase enzyme deficiency, which appears with the accumulation of glucocerebrosides in the cells and certain organs of the patient body (4). These therapeutic enzymes were produced by recombinant DNA technology through introducing the related enzyme genes into the host cell.

One of the recent molecular tools for genetic manipulation of host genome is transposon system. Transposons are mobile genetic elements that influence the structure and functions of the genome, especially in the evolution of organism (5). The transposons consist of class I and II based on transposition mechanisms. Class I elements use a RNA intermediate and reverse transcriptase by a "copy-and-paste" mechanism for transposition, whereas Class II mobility is done through DNA mediated via a "cut-and-paste" mechanism. Class II elements encode a transposase that can recognize specific inverted terminal repeat sequences (ITRs) located on both sides of the transposon, then excises sequence between ITRs and inserted into another sites. The parental sequence from a donor site and reintegrates it into another location in the genome (6). Genetic engineering strategy based on transposon rely on class II mobilizing two-component system. For this purpose, the gene of interest is inserted between two ITRs in the transposon vector, and the transposase removes this GOI from the donor vector and integrates into other locations of the host genome (7). Sleeping Beauty (SB), Tol2 and PiggyBac (PB) transposon system are well recognized tools for genetic manipulation (8).
Among these, PiggyBac transposon system (PB) isolated from the cabbage looper moth *Trichoplusia ni* genome (9) was chosen because of its several useful features. It is active in the mammalian cells for long term expression (10, 11) precise excision ability (12) and large cargo capacity over than 100 kb (13). PB can recognize TTAA segment in the whole genome of the mammalian cells and inserted into these sites. The length of the PB mobile element is 2427 bp with specific 13 bp inverted terminal repeat sequences (ITRs). PB transposase consists of 594 amino acids with a molecular weight of 68 kDa (14). This transposon system has been used for the production of recombinant proteins in mammalian cells such as Chinese hamster ovary (CHO) cells (15). The common platform for the recombinant therapeutic protein expression in the mammalian cells is CHO cells (16). It is due to post-translational modifications ability in CHO cells for correct glycosylation of recombinant product proteins (17).
In this work, we used PiggyBac transposon system for expression of elosulfase alfa and imiglucerase in the CHO cells. We constructed transposon vectors with GFP, GALNS and GBA genes separately and then introduced these vectors together with transposase into the CHO cells.
The success of gene integration into the host genome via the transposon system was confirmed by monitoring GFP expression. After that, the recombinant human GALNS and rhGBA cell lines generated by this method were able to express the recombinant enzymes.

Material and Methods

Plasmids

For introducing desired gene into the host genome, two vectors including transposas (SuperPBase) and PB transposon were designed according to the PiggyBac transposon system. First, GALNS gene (NCBI Gene ID: 2588) along with SUMf1 gene (NCBI Gene ID: 285362) construct were digested by NheI/BstbI restriction enzymes at the compatible site with PB transposon vector, then two fragments were ligated by T4 ligase (Thermo Fisher, US) to form PB::GALNS vector. Similarly, GBA (NCBI Gene ID: 2629) construct and PB vector by NheI/BstbI restriction enzymes were digested and ligated to create PB::GBA vector. As well as PB::GFP control vector were constructed by ligation between NheI/NaeI digested GFP fragment and NheI/AfeI linear PB fragment.

Cell culture

The HamF12 medium (Biowest, France) supplemented with 10% Fetal Bovine Serum (Biowest, France) were used for the CHO-K1 cell (ATCC-CCL-61) culture in incubator with 5% C02 and 95% humidity, at the temperature of 37 °C. The recombinant GALNS and GBA cell lines were generated by genetic engineering of CHO-k1 cells.

Recombinant cell lines

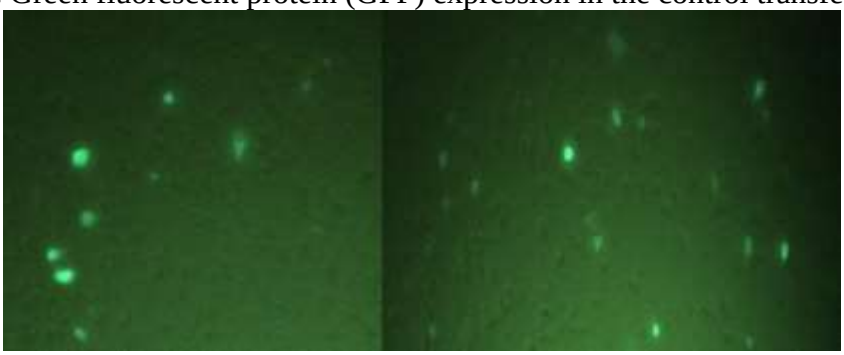
For GALNS recombinant cell line development, CHO-k1 cells were cultured and plated at the density of 0.2–0.3 $\times 10^6$ cells/mL in a 12-well plate (Nest, Germany). After 24 hours, transfection by lipofectamine 2000 reagent (Thermo Fisher, US) was performed using PB::GALNS vector and transposas vector. In the next step recombinant GALNS cells were selected by addition of Zeocin (Thermo fisher, US) at 400 μg/ml concentration.

Generation of GBA recombinant cell line was carried by PB::GBA vector and transposas vector transfection to CHO- k1 plated cells at 0.2–0.3 $\times 10^6$ cells/mL density. Then transfected cells were exposed to Zeocin (Thermo fisher, US) at 400 μg/ml concentration for GBA recombinant cell line selection. In both transfection process, GFP control cell lines were created by PB::GFP and transposas vectors usage.

Verification of integration and expression

GALNS and GBA recombinant cells were harvested and their genomic DNA were isolated by DNA Extraction kit (Favorgen, Taiwan). Polymerase Chain Reaction (PCR) were performed by Fe: CCTGACACAGATCTACCTGC and Re: CGTCGATCTCTCTAACAGCG specific primers for GALNS gene integration and Fi: CAGAAGGTGAAAGGCTTCGG and Ri: GTGCAG TTGGAAGTCGTCAG for verification of GBA insertion gene into CHO genome. Expression of elosulfase alpha enzyme in rhGALNS cell line and imiglucerase in rhGBA cell line were verified by sandwich ELISA kit.

Figure 1. Green fluorescent protein (GFP) expression in the control transfected cells



Results

GFP positive cells appeared when CHO-k1 cells were transfected with PB::GFP and transposase vectors (Figure 1). These results indicated that designed transposon system with PB and SuperPBase works very well and GFP gene was integrated into the CHO genome by transposase. Furthermore, GFP transposon used as a control vector in the transfection process to ensure the correctness and efficiency of the steps.

Expression of elosulfase alfa enzyme (GALNS)

Simultaneously with GFP positive cells appearance as a control, in the next well, transfected cells by PB::GALNS / SuperPBase vectors were investigated to confirm elosulfase alfa expression in the recombinant cell lines. For this, the whole plated cells exposed to zeocin were washed and only the surviving cells were cultured again. This was while the non-transfected cells in the same plate as a negative control were all destroyed when exposed to zeocin. Then the transfected survive cells were

cultured to create recombinant cell pools. At the end, the supernatants of the expanded cell lines were harvested and analyzed by ELISA. Sandwich ELISA with specific antibody to Human GALNS verified expression of elosulfase alfa in the recombinant cell lines. PCR data analysis showed that GALNS gene inserted into the CHO genome.

Expression of imiglucerase (GBA)

In other well of the plate, CHO cells were transfected by PB: GBA/SuperPBase to produce imiglucerase enzyme in the cell culture. Similar to the previous screening method, living cells were cultured after being exposed to zeocin, and the expression of imiglucerase verified in recombinant GBA cell lines by ELISA method. Investigation of genome of GBA recombinant cells by PCR confirmed integration of GBA gene.

Discussion

One of the efficient nonviral DNA delivery tools for genetic manipulation is PiggyBac transposon system due to its unique features, higher safety than viral vectors without immunogenic and tumorigenic potency (18). Generation of a recombinant PB vector with gene of interest is convenient (19). As well as, the large cargo size (9.1–14.3 kb) in transition by PB and high transposition activity are the advantages of this system compared to other transposons (20, 21). In the several previous studies, PiggyBac transposon has been used to rapid create recombinant CHO cell pool to produce the desired products. These recombinant CHO cell lines were stable with high production potential (15, 22, 23).

In this work, we used PB system for manipulating of CHO genome as a host cell to produce a recombinant enzyme. In the first step, the performance of the designed transposon system was confirmed by the appearance of fluorescent green cells, then this system was used to express therapeutic enzymes in CHO cells. Elosulfase Alfa and imiglucerase are two therapeutic enzymes that, due to their structural complexity, their optimal production process requires a suitable host cell line and a strong molecular tool for gene of interest integration.

For Elosulfase Alfa production, simultaneous expression of the auxiliary enzyme SUMF1 is required. SUMF1 (Sulfatase modifying factor 1) encodes a formylglycine-generating enzyme that activate human sulfatases such as GALNS by conversion of cysteine residue to c-alpha formylglycine (24, 25). Expression of SUMF1 enzyme along with GALNS can increase enzyme activity of GALNS (26). Therefore, the size of the designed gene construct for the simultaneous expression of these two enzymes will be large which the use of the PB transposon system in this study enables its transfer. Also, several copies of the target gene are integrated into the CHO host genome. Then transposon system was appropriate tool for generation of rhGALNS and rhGBA cell lines.

Conclusion

In conclusion, The PiggyBac transposon system can be considered as an effective molecular tool in the modification of the host genome to generation of recombinant cell lines with the ability to carry large gene constructs. In addition, precise integration of several desired gene copies in the host genome can increase the production of recombinant proteins.

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