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Research Article

Construction of the Recombinant Lentiviral Vector Containing Human *GH1* Gene and its Expression in HEK293T Cells

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Abstract

Human growth hormone (*hGH*) is a protein with multiple roles in a range of biological functions such as protein, carbohydrates and lipid metabolisms as well as immunity, tissue development and overall growth. One of the major class of biopharmaceuticals in mammalian cells is the production of recombinant pharmaceutical proteins. In this study, we constructed a lentiviral vector carrying coding region of human GH1 (*hGH*) gene in order to production of recombinant *hGH* in mammalian cell line. *hGH* gene was amplified from a plasmid containing full-length *hGH* coding sequence and then cloned into the lentiviral vector pCDH-GFP. The HEK293T cells were transduced by the lentivirus particles as a targeted cell. *hGH* expression status in the recombinant cells were confirmed by RT-PCR. Additionally, western blotting analysis results showed that the recombinant cells maintained a stable *hGH* expression during five weeks of continuous culture. In conclusion, results of current study suggested that constructed lentiviral vector can potentially be used for a stable production of recombinant *hGH* protein in HEK293T cells. This methodology could be served as a foundation for further research and may open new insights toward therapeutic protein manufacturing.

***Keywords*: *hGH*, Recombinant lentivirus, Production protein, HEK 293 cells**

Introduction

There are large numbers of *in vitro* and *in vivo* studies proofing that *hGH* gene is a major regulator of growth (Levy, 2000). The growth hormone is a member of the somatotropin family (Yi et al., 2002) and currently, recombinant *hGH* has some therapeutic applications in the treatment of AIDS, dwarfism, bone fractures, skin burns and bleeding ulcers (Velloso, 2008).

Recombinant *hGH* is a protein that made to be very similar to the human growth hormone that is naturally active. This recombinant protein can enhance cell and tissue growth, linear growth (height), and metabolism of proteins, carbohydrates, lipids and minerals (Rezaei et al., 2012). Several expression systems have been established for recombinant protein production such as bacterial, yeast, insect and mammalian cells (Mao et al., 2015). Bacterial systems are the oldest and most extensively used expression systems, they are not appropriate to express eukaryotic proteins because of loss of correct post-translational modifications essential for full biological activity (Baneyx and Mujacic, 2004). Yeast expression systems often achieve higher yield than bacterial systems, and have the ability to express more complex proteins and conduct post-translational modifications necessary for complete biological activity (Daly and Hearn, 2005). Insect cell systems have become popular for recombinant protein production due to both short process development time and potential high yields. While, the glycosylation of insect cells is significantly different from mammalian cells because they are believed to be unable to process complex-type oligosaccharides (Kost et al., 2005). Expression systems based on mammalian cell lines for producing recombinant proteins have the capacity to perform different post-translational modifications and correct protein folding which are critical for biologically active proteins (Khan, 2013). Mammalian cells can express recombinant proteins through transient transfection and viral transduction (Gaillet et al., 2010). Transient expression systems

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are not stable and may lead to loss of expression, therefore they are not efficient in addition to high costs of production. In contrast, lentivirus vectors have the ability to integrate into the nucleus of the target cells and provide stable expression of transgene in long term (Nayerossadat et al., 2012) when abundant amounts of material have to be generated on a routine basis (Lundstrom, 2003). This requires an expression system based on virus or on producing cell lines which have a stable integration of the inserted DNA in the host's genome. However, the integration of the recombinant constructs inside the host's genome, certainly result a wide range in protein synthesis levels within the same batch of cells (Assur et al., 2012). Considering the vast therapeutic applications of *hGH* and the approved successful application of lentivirus in transferring genes into mammalian cells, the goal of this study was, cloning the *hGH* gene into lentiviral vector and production of recombinant virus in HEK293T (human embryonic kidney) cells with the ability of expression of the *hGH* protein.

Materials and Methods

Cell Cultures

HEK293T cells (ATCC CRL-3216) (obtained from Mede Bioeconomy Company, Iran) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), supplemented with 10 % fetal bovine serum (FBS) (Gibco, USA), 100 units/ml penicillin, 100 mg/ml streptomycin (Invitrogen, USA) and incubated at 37C° in a humidified 5% CO_2 atmosphere.

Construction of hGH-Expressed Recombinant Lentivirus Vector

To amplify *hGH* sequence, a pair of primers (forward primer, 5´- **TCT AGA** ATG GCT ACA GGC TCC CGG -3´; reverse primer, 5´- **GCG GCC GC**C TAG AAG CCA CAG CTG CCC TC -3´) were designed and synthesize based on the *hGH* cDNA sequences (NM-000515.3). They had contained both *Xba*I and *Not*I restriction enzyme cutting sites (bold). The polymerase chain reaction (PCR) was used to amplify the *hGH* sequence using pUC57 plasmid contains *hGH* as a template. The thermal cycling condition was: 95 °C pre-denaturation for 5 min; 95 °C denaturation for 30 s, 56 °C annealing for 30 s, 72 °C extension for 30 s for 30 cycles; 72 °C extension for 7 min. The PCR products were electrophoresed in a 1% agarose gel, and ~550 bp *hGH* product was purified and cloned into the shuttle plasmid pCDH- GFP to construct the pCDH-*hGH*-GFP lentivirus vector. In order to confirm the clone of containing recombinant plasmid, enzymatic digestion was performed with *Xba*I and *Not*I enzymes and finally the recombinant pCDH- *hGH*- GFP plasmid was sequenced.

Production of hGH-expressed Recombinant Lentivirus Particles

The lentiviral vector were transfected with three plasmids pCDH- GFP, psPAX2 and pMD2.G (a gift from Tronolab) in HEK-293T cells, using the calcium phosphate method with some modifications (Trono, 2000; Roudbari et al., 2015). On day one, 5×106 HEK-293T cells were seeded in a 10 cm plate in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) with 10% FBS (Gibco, USA). On the second day, 21 µg of transfer vector, 21 µg of psPAX2 vector and 15 µg of pMD2.G vectors were mixed with transfection buffer and added drop-wised to the cells. Transfection medium was replaced with fresh medium within 14 hours post transfection. The expression of GFP was determined after 24 hours by fluorescent microscopy. The packaged recombinant lentiviruses were harvested from the supernatant of cell culture after 24, 48 and 72 hours post transfection. Then centrifuged at 2000 rpm at 4°C for 5 min and the supernatant filtered through a 0.22 µm filter. The recombinant virus was stored at -70°C for subsequent experiments. Viral titer was determined with counting the number of GFP positive cells by flow cytometry.

Transduction of HEK293T Cells

HEK293T cells at a concentration of 2×10^5 were seeded in a 6-well plate. The cells were transduced on the following day with recombinant lentivirus at a multiplicity of infection of 20. After 16 hours, transduction media was replaced with fresh DMEM containing 10% FBS. The transduced cells were passaged every three days. Transduced cells were assayed for GFP expression with a fluorescent microscope at 72 hours after infection and weekly for 5 weeks. Images were evaluated by Image J software (Jensen, 2013). GFP expression indirectly indicated expression of hGH and transduction efficiency of the recombinant lentivirus was assessed by flow cytometry.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

After 7 days of infection, total RNA was extracted from cells using RNeasy ® Plus Mini Kit (Qiagen, Germany). Complementary DNA (cDNA) was synthesized using QuantiTect Rev Transcription Kit (Qiagen, Germany), according to manufacturer instruction. RT-PCR conditions were as follows: a

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cycle of 95°C for 5 min, and 30 cycles of 95°C for 30 s, 54°C for 30 s and 72°C 30 s and a cycle of 72°C for 7 min. The primer sequences were used to RT-PCR are listed in Table 1. PCR products were run on 1.5% agarose gel and stained with DNA safe stain (Sinaclone, Iran). Gel images were visualized with a UV transilluminator (SABZ biomedical, Iran) and the integrated optical density (IOD) of each band was measured by Image J sowftware. The levels of *hGH* mRNA were normalized against *β-actin*.

Table1. Primer sequences for RT-PCR.

Target gene	Primer sequences	Amplification length (bp)
***h*GH-F**	TAGAATGGCTACAG GCTCC	183
***h*GH-R**	GCTTCTTCAAACTCC TGGTAG	
β-actin-F	AGCCTCGCCTTTGCC GA	172
β-actin-R	CTGGTGCCTGGGGCG	

Western Blotting Analysis

From week-1 to week-5 after infection, the cells weekly were lysed in 200 µl of RIPA buffer (Thermo Fisher Scientific, USA) supplemented with protease inhibitor. The cell lysates were centrifuged at 11000 rpm for 12 min at 4ºC and the supernatants were collected. Protein concentrations of the supernatants were determined with a BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Protein lysates (30 µg/lane) were loaded onto 12% SDS–PAGE gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, USA). The membranes were blocked with 5% non-fat dried milk and immunoblotting was performed with *hGH* (abcam, UK) and β-actin (Santa Cruz, USA) antibodies at 1:1000 dilutions. Anti-mouse IgG monoclonal antibody, conjugated with horseradish peroxidase at 1:2000 dilution. Finally the protein band was visualized by chemoluminescence reagent (ECL) and the integrated optical density (IOD) of each protein band was measured. IOD values were adjusted by internal standard β-actin. The concentration of recombinant *hGH* protein was determined by SDS-PAGE/Densitometry using ImageJ software.

Statistical Analysis

All experiments were conducted in triplicate and values were represented as mean ± SD. Statistical differences between groups were compared by one-way analysis of variance (ANOVA) using the SPSS 20.0 software. $P<0.05$ was considered statistically significant.

Results

Construction of Lentiviral Vector Containing *hGH* Gene

The coding sequence of *hGH* with 550 bp was successfully cloned into the pCDH- GFP vector and the recombinant lentiviral vector was named pCDH-hGH- GFP.

Sequencing results confirmed that the cloned *hGH* sequence was amplified and inserted correctly (data not shown). Digestion with *Xba* I and *Not* I enzymes also verified the accuracy of cloning (Figure 1).

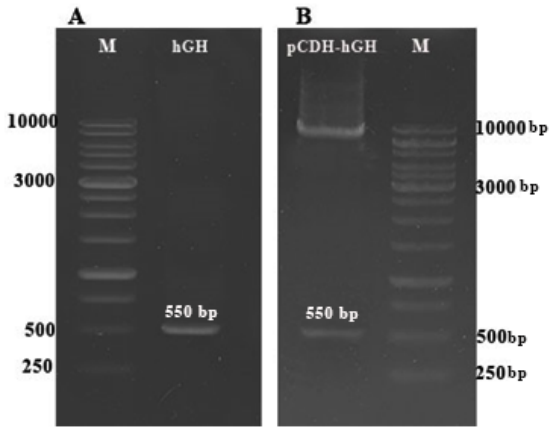

Figure 1. Construction of *hGH* recombinant lentiviral vector. A: PCR product of *hGH* and B: Recombinant pCDH-*hGH* digested by restriction enzymes *Xba* I and *Not* I, lane M: 1 kb DNA marker.

Packaging and Titration of The Recombinant Lentivirus Vector

The packaging of the recombinant lentivirus was verified using the expression of GFP by fluorescence microscope 24, 48 and 72 h after transfection. As shown in Figure 2, more than 90% of HEK293T cells were transduced by the lentivirus pCDH-hGH-GFP.

The lentivirus titer determination was based on the expression of GFP in a cell-based assay. The titer of the recombinant virus was approximately 1×10^7 IU/mL.

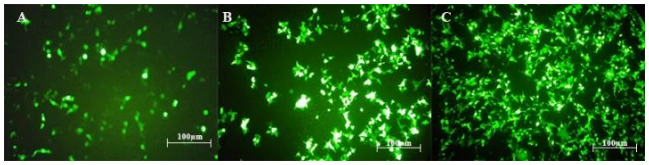

Figure 2. Fluorescent microscopy image showing GFP expression of HEK293T for achieving viral particles by pCDH- *hGH*. A: HEK 293T at 24 hours after transfection, **B**: HEK 293T at 48 hours after transfection and **C**: HEK 293T at 72 hours after transfection.

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Recombinant Lentivirus Mediated GFP Expression in HEK293T Cells

Transduction efficiency of the recombinant lentivirus was initially investigated by analyzing GFP expression in HEK293T cells. The number of GFP-expressing cells and GFP intensity was assessed 3 days post transduction. Typically, at a multiplicity of infection of 20, more than 90% of the transduced cells were found to be GFP-positive using fluorescence-activated cell sorting analysis (Figure 3A and 3B). Therefore, HEK293T cells were successfully transduced with recombinant lentivirus with a high efficiency. Tracing transduced HEK293T cells with fluorescent microscope for 5 weeks showed that the percentage of GFP positive HEK293T cells did not change and GFP stably expressed (Figure 3C and 3D).

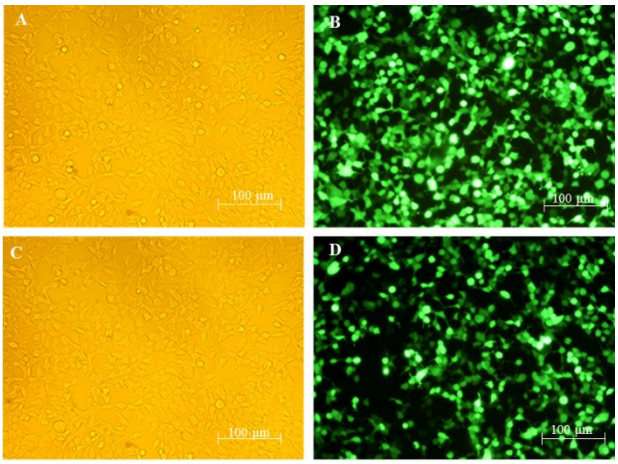

Figure 3. Transduction of HEK293T cells by recombinant lentivirus particles. A, B: Transduced HEK293T before and after fluorescent illumination; 72 h after transduction and C, D: Transducted HEK293T before and after fluorescent illumination; 5 weeks after transduction

The mRNA Expression of *hGH* in HEK293T Cells Using RT-PCR

The mRNA expression of *hGH* was measured using RT-PCR. The expression level of *hGH* gene was normalized to the expression of the β-actin gene, as reference gene. Based on the results of comparative analysis with Image J software, the transcription of *hGH* was detected in cells transduced with pCDH-*hGH* recombinant construct. There was no detectable transcript of *hGH* in HEK293T cell transduced by the pCDH-GFP (negative control) and non-transduced cells (Figure 4). The expression of *hGH* at mRNA level suggested that the pCDH-*hGH* lentivirus functionally integrated into the genome of HEK293T cells.

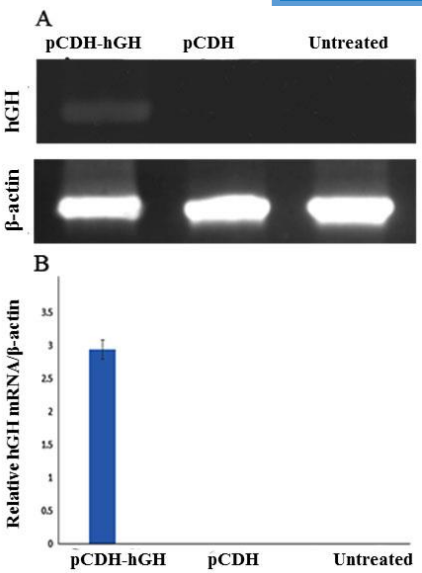

Figure 4. Effect of *hGH*-expressing recombinant lentivirus on the mRNA expression of *hGH* in HEK293T cells. A: RT-PCR analysis of different HEK293 cell groups after transduction. B: The *hGH* mRNA expression levels of different HEK293T cell groups were calculated using β-actin as an internal standard. (Data are means ±SD).

hGH Protein Expression in HEK293T Cells Using Western Blot

In order to confirm the expression of hGH protein in recombinant HEK293T cells western blot was performed. The result of immunoblotting analysis demonstrated that hGH protein was produced only in transduced HEK293T cells by hGH-expressed recombinant lentivirus (Figure 5).

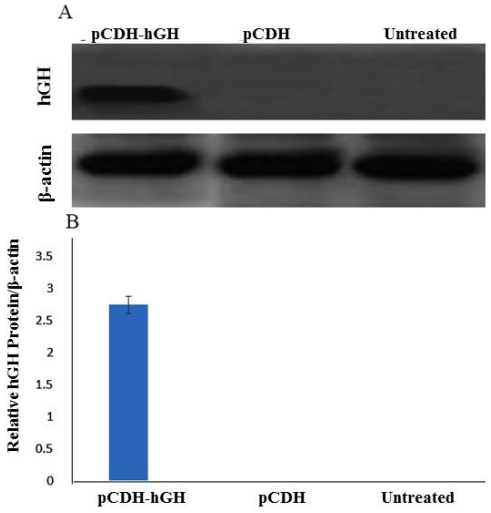

Figure 5. Effect of *hGH*-expressing recombinant lentivirus on the protein expression of *hGH* in HEK293T cells. A: Western blots analysis of different HEK293 cell groups. B: The *hGH* protein expression levels of different HEK293T cell groups were calculated using *β-actin* as a loading control. (Data are means ±SD).

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Also results showed, recombinant HEK293T cell line maintained robust *hGH* expression for at least 5 weeks without any significant change in expression of gene (Figure 6). The concentration of recombinant hGH protein was calculated as 0.23 mg/ml by using densitometry analysis of band intensities from SDS polyacrylamide gel electrophoresis (SDS–PAGE) in Image J software.

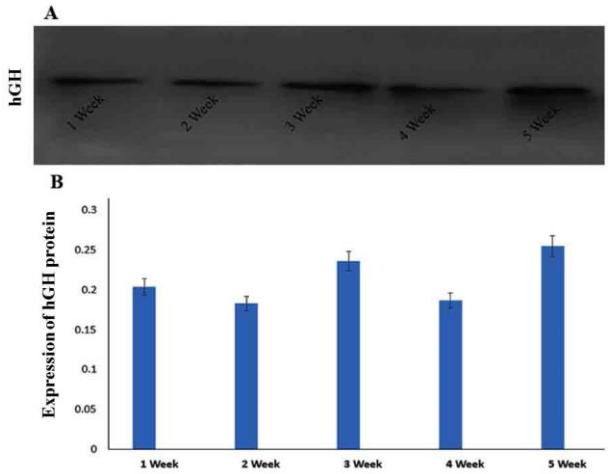

Figure 6. The sustained expression of *hGH* in transduced HEK293T cells by pCDH-*hGH* lentivirus. A: representative western blot showing sustained expressing of *hGH* from 1 week to 5 weeks. B: Bar graphs showing the expression of *hGH* levels in each time point. (Data are means ±SD).

Discussion

Recombinant pharmaceutical proteins are one of the most expensive and important therapeutic productions of the present time, and the production of recombinant proteins in mammalian cells represent a major class of biopharmaceuticals (Merlin et al., 2014).

Recent studies have demonstrated high efficiency for lentivirus transgenesis in mammalian cell lines including HEK293T and CHO (Huh et al.,2007). These cell lines can be widely used for production of recombinant proteins by viral-mediated transduction as well as by the formation of stable cell lines (Bell et al., 2015).

In the present study, we successfully constructed a lentiviral vector expressing *hGH* gene verified by restriction enzyme digestion and sequencing. This recombinant lentivirus had a bright form of green fluorescent protein (GFP): copGFP.

Numerous studies have shown that copGFP as a new version of GFP with boosted fluorescence that is more useful for enhanced visualization in vivo and in vitro (Day and Davidson , 2009; Alizadeh et al.,2015).

In current study, GFP was very functional in analysis of transfection efficiency in HEK 293T cells after packaging to produce recombinant viruses and assessment of transduction efficiency in transduced HEK293T cells. The results of fluorescent microscopy showed that HEK293T cells after transduction with recombinant lentiviruses expressed GFP stably during this experiment (5 weeks in this study).

It has been approved that compared with traditional non-viral shuttle vectors, Viral vectors have been widely used as shuttle vectors in recent studies due to their high efficiency in delivery, quick and long term effect and cost effective (MenezesK et al., 2006). Ma et al (2006) evaluated the efficiency of transfection of marrow stem cells with lentiviral vector and adenoviral vector, and they reported that the transfection efficiency of desired gene by lentivirus is considerably higher in compare to adenovirus.

As part of this study, we showed that the recombinant lentivirus could stably produce recombinant proteins in transducted cells after five weeks. In comparison with transient transfection, where production happened only in a short period (2-6 days) (Subramanian and Srienc, 1996), our results showed that the protein expression of hGH was stable for at least five weeks.

This fact was confirmed with the finding of Liu et al where they constructed a recombinant lentivirus vector mediated hGH in skeletal muscle myoblasts and results indicated long, efficient and stable expression of the recombinant hGH in skeletal muscle myoblasts (Liu et al., 2006). In another study, it was shown that the expression level of the desired protein by lentivirus was stable for at least 9 weeks (Mao et al., 2015).

The concentration of hGH protein in the current study showed that transduced HEK293T cells by recombinant lentivirus could approximately produce hGH with an increase of twice higher yield than E. coli which were transformed by plasmids containing hGH gene (Rezae and Zarkesh-Esfahani, 2012). In summary, the hGH-expressed by recombinant lentivirus evaluated in this study stably transduced HEK293T cells and resulted in long term expression. These findings provide valuable perception that could improve experiments leading to recombinant protein manufacturing especially in HEK 293T cells. The lentivirus expressing system is a highly efficient and also a simple gene transfer method. To our knowledge, this methodology could be served as a foundation for our further research and may be adapted for therapeutic protein manufacturing.

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