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Retrovectors packaged in CHO cells to generate GLP-1-Fc stable expression CHO cell lines

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ABSTRACT

Background: Chinese hamster ovary (CHO) cells are the most dependable mammalian cells for the production of recombinant proteins. Replication-incompetent retroviral vector (retrovector) is an efficient tool to generate stable cell lines. Multiple copies of integrated genes by retrovector transduction results in improved recombinant protein yield. HEK-293 and their genetic derivatives are principal cells for retrovector production. Retrovectors packaged in HEK-293 cells pose a risk of infectious agent transmission, such as viruses and mycoplasmas, from serum and packaging cells.
Results: In this report, retrovectors were packaged in CHO cells cultured in chemically defined (CD) media. The retrovectors were then used to transduce CHO cells. This method can block potential transmission of infectious agents from serum and packaging cells. With this method, we generated glucagon-like protein-1 Fc fusion protein (GLP-1-Fc) stable expression CHO cell lines. Productivity of GLP-1-Fc can reach 3.15 g/L. The GLP-1-Fc protein produced by this method has comparable bioactivity to that of dulaglutide (Trulicity). These stable cell lines retain 95–100% of productivity after 40 days of continuous culture (~48–56 generations).
Conclusions: Suspension CHO cells are clean, safe, and reliable cells for retrovector packaging. Retrovectors packaged from this system could be used to generate CHO stable cell lines for recombinant protein expression.
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1. Introduction

Chinese hamster ovary (CHO) cells are frequently used as hosts for the production of recombinant therapeutics because of their high yields of proteins, suspension culture in serum-free or chemically defined (CD) media, similar post-translational modifications to human cells, and established regulatory and safety profile. A key step in therapeutic recombinant protein development is to generate a monoclonal cell line that consistently expresses the given protein with high quantity and quality.

Traditionally, cell line development starts with vector transfection followed by antibiotic selection, gene amplification mediated by the DHFR (dihydrofolate reductase) or GS (glutamine synthetase) system [1], single-cell cloning, and evaluation of single-cell clones. It is a laborious and time-consuming process that normally takes 5 to 8 months. DHFR/MTX and GS/MSX gene amplification systems could amplify gene copies up to several hundred-fold. However, amplified genes are unstable, prone to be lost by homologous recombination [2] or be silenced by epigenetic modifications such as DNA methylation [3], heterochromatin formation [4], or subsequent mutagenesis [5]. Replication-incompetent retrovectors could integrate multiple copies of viral cDNA in a stable manner into the transcriptionally active region of host cells' chromosomes with an efficiency of approaching 100% for actively dividing cells. The high transduction efficiency and integration of multiple copies eliminate requirement for gene amplification. Replication-incompetent retrovectors genetically modified from murine leukemia virus (MLV) have been used for the generation of stable cell lines for therapeutic protein development. Some monoclonal antibodies and Fc-fusion proteins produced from CHO cell lines generated with Catalent's GPEx (MLV-based retrovector) are under commercial development, such as Pritumumab, an antibody for the treatment of glioma [6,7]. In the GPEx system, retrovectors were packaged in adherent HEK-293 cells in a serum-containing medium.

Adherent HEK-293 cells require the presence of bovine serum in their growth medium. Serum is a potential source of contamination by viruses, mycoplasmas, and even prions. It has been reported that Parainfluenza virus, Reovirus, Bovine adenovirus, Bovine parvovirus,

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Bovine respiratory syncytial virus, Bovine viral diarrhea virus, Rabies virus, Vesicular stomatitis virus, Bluetongue virus, and Foot and mouth disease virus could be isolated from bovine serum [8]. To avoid contamination by potential infectious viruses from serum, retrovectors could be packaged in suspension HEK-293 cells cultured in a serum-free or CD medium. However, mammalian cell lines are optimal reservoirs for various viruses [9,10]. Many viruses could replicate in HEK-293, such as Adenovirus type, Adeno-associated virus type, Bovine Adenovirus, Human Parvovirus B19, Bluetongue virus, Bovine parainfluenza virus, Bovine respiratory syncytial virus, Corona virus, Epstein–Barr virus, Human herpes virus, and Rhinovirus [11,12,13]. The source of these viruses is laboratory operators, environment, and any animal-sourced materials such as serum and trypsin. Retrovectors from HEK-293 cells contaminated by the viruses mentioned above will definitely introduce infectious viruses to CHO cells. Many of the viruses listed above could replicate in CHO cells, such as Adenovirus, Parainfluenza virus, Bovine respiratory syncytial virus, Vesicular stomatitis virus, Reovirus, and Bluetongue virus [14]. Furthermore, cell lines contaminated by viruses cannot be treated to become virus free. HEK-293, either adherence or suspension, still pose risk of transmitting infectious viruses to CHO cells.

In this study, we packaged retrovectors in suspension CHO-S cells cultured in a CD medium. Harvested retrovectors were used to transduce CHO-S cells. With this method, we eliminated potential infectious agents from traditional retrovector packaging cell line and generated stable cells expressing glucagon-like peptide-1 Fc fragment fusion protein (GLP-1-Fc), a long-acting GLP-1 analog for type 2 diabetes. The yield of GLP-1-Fc recombinant protein can reach 3.15 g/L. GLP-1-Fc produced from this method has comparable bioactivity to that of commercial GLP-1-Fc (dulaglutide).

2. Materials and methods

2.1. CHO cell culture

Freedom CHO-S (Cat. No. A11364-01, cGMP banked) cells were purchased from Thermo Fisher Scientific (Waltham, MA, USA). CHO-S cells were maintained in CD CHO medium (Cat. No. 10743, Gibco) or CD FortiCHO medium (Cat. No. 12681, Gibco). These CD media were supplemented with 8 mM L-glutamine (Cat. No. A2916801, Gibco). Viable cells were counted with a Countess Automated Cell Counter (Cat. No. C10277, Invitrogen). CHO cells were passaged at 2×10^5 viable cells/mL every 3 days or 3×10^5 viable cells/mL every 2 days. Dynamis medium (Cat. No. A2661501, Gibco) was used for fed-batch culture.

2.2. Plasmids

pCMV-gag-pol and pMD2.G (GenBank: MS855620.1) were kindly provided by Dr. Frank J. Rauscher (The Wistar Institute, Philadelphia, USA). A retroviral transfer vector pLEGFPC1 (coding for green fluorescent protein) was used as a positive control. To obtain a high and stable recombinant protein production in CHO cells, a retroviral transfer plasmid, pLPCX (Clontech), was modified by replacing the human CMV promoter with the murine CMV promoter (mCMV) upstream of the multiple cloning site (MCS) and insertion of a woodchuck post-transcriptional regulatory element immediately downstream of the MCS. The new plasmid was designated as pRDM. A GLP-1-Fc expression cassette was synthesized by GenScript (Nanjing, China) and subcloned into pRDM to obtain pRDM-GLP-1-Fc. All constructs were confirmed by restriction enzyme digestion and DNA sequencing.

2.3. Retrovector production

To produce retrovectors for CHO-S transduction, a transfer plasmid (pRDM-GLP-1-Fc or pLEGFPC1) was transfected together with the packaging plasmids pMD2.G and pCMV-gag-pol, using Freestyle Max Reagent (Cat. No. 16447, Invitrogen). Twenty-four hours post-transfection, cells were treated with 10 mM sodium butyrate for 12 h. Then cells were centrifuged and re-suspended in fresh CD FortiCHO medium. Viral supernatant was harvested at 48–60 h post transfection and filtered through 0.45 μm filters. Polybrene was then added into the viral supernatant at a final concentration of 4 μg/mL to enhance transduction efficiency.

2.4. CHO cell transduction and stable cell line generation

2×10^5 cells were inoculated into 1 mL viral supernatant (multiplicity of >100 retrovector particles/cell) and incubated at a shaker incubator for 3–4 h at 37°C, 8% CO_2, 110 rpm, and 95% humidity. Then, four volumes of the fresh CD FortiCHO medium were added. Forty-eight hours post-transduction, the CHO-S cells were selected with 7.5 μg/mL puromycin for 4 days. The survived cells were used for fed batch or limiting dilution cloning. Repeated viral transductions could be performed to increase protein productivity.

2.5. Limiting dilution cloning

CHO-S cells were seeded at a density of 0.3 cell/well on 96-well plates (Cat. No. 353072, Corning) in the CD FortiCHO medium containing 6 mM L-glutamine. Cells were then incubated undisturbed for 12–14 days at 37°C, 5% CO_2, and 95% humidity in a static incubator. After 12–14 days, single-cell colonies are transferred from 96-well plates to 15 mL mini-bioreactors (TubeSpin, TPP) for further culture.

2.6. Fed-batch culture

The shaking flask fed-batch culture experiments were performed in 250 mL shaking flasks (Cat. No. 431144, Corning) with a 50 mL working volume at a seeding density of 5×10^5 cells/mL. Dynamis medium was used for the fed-batch culture. Cultures were agitated at 120 rpm in an incubator operated with 8% CO_2 at 37°C and 95% humidity. Efficient Feed C+ supplement (Cat. No. A1327501, Gibco) solution was added on days 3, 5, 7, and 10 at volumes of 10% of the initial working volume. Temperature was shifted to 33°C when cell concentrations reached $15–20 \times 10^6$ cells/mL. Cultures were harvested on day 14 or when viability dropped below 70%. Glucose, lactate, glutamine, and ammonium concentrations were measured daily using a Cedex-Bio analyzer (Roche, Switzerland). Osmolality was measured daily using an Osmomat 030-D cryoscopic osmometer (Gonotec, Berlin, Germany).

2.7. Size-exclusion chromatography (SEC)

GLP-1-Fc protein concentration was determined using a Waters Alliance 2695 equipped with a 2489 UV/Visible detector. A TSK gel G2000SWXL column (5 μm diameter particle, 7.5 × 300 mm) was purchased from Tosoh. Briefly, GLP-1-Fc stable expression CHO-S cells and CHO-S cells (negative control) were fed-batch cultured in 250 mL shaking flasks with 50 mL medium for 14 days. Culture media were harvested by centrifugation at 10,000 rpm, 4°C, for 15 min. The supernatants were filtered through 0.22 μm filters (PN4612, Pall) to further separate particulate matter. Dulaglutide was used as a reference standard (0.75 mg/0.5 mL, Eli Lilly). Ten microliters of filtered cell culture supernatants and dulaglutide injection solution were injected separately into the column. 0.01 M PBS (145.45 mM NaCl, 9.86 mM Na_2HPO_4 and 1.67 mM NaH_2PO_4, pH 7.2) was used as the mobile phase. Column temperature was 30°C. The runtime was 70 min at a flow rate of 0.5 mL/min for each sample. Data were collected at 214 nm. Peaks were identified by comparing with the retention time and spectra of dulaglutide. The retention time of the dulaglutide molecule was 13–15 min. For GLP-1-Fc stable expression CHO-S supernatants, peak with retention time identical to that of

dulaglutide (13–15 min) was considered mainly GLP-1-Fc. For CHO-S supernatants, peak with a retention time of 13–15 min was considered as background. Quantitation of peak area was performed with Chromeleon 7 software according to the user manual. Peak area (GLP-1-Fc) was normalized with that of dulaglutide to obtain GLP-1-Fc concentration.

2.8. Cell line stability evaluation

Single-cell clones were passaged without selection pressure for 40 days in the CD FortiCHO medium. Briefly, cells were passaged at 2×10^5 cells/mL every 3 days for 40 days. The productivity of generation 1 (Day 1) was compared to cells that of complete 40-day (Day 40) continuous cultivation. Fourteen-day shaking flask fed-batch culture was performed for GLP-1-Fc productivity assay.

2.9. In vitro bioactivity assay

cAMP response element (CRE)-driven GFP assay was performed with RIN-m5f/CRE-GFP cells that harbor the CRE-GFP cassette. Protein A-purified GLP-1-Fc was compared with Trulicity (dulaglutide, 0.75 mg/0.5 mL, Eli Lilly). Briefly, exponentially growing RIN-m5f/CRE-GFP cells were stimulated (in triplicate) with either serially diluted dulaglutide or GLP-1-Fc for 6 h. GFP was measured with a fluorescence plate reader (Synergy H1M, Biotek) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The four parameter logistic equation was used to determine the EC50 value.

2.10. LC–MS/MS peptide mapping

Dulaglutide and protein A-purified GLP-1-Fc were digested with IdeS (Cat. No. V7511, Promega), which specifically cleaves IgG molecules below the hinge region to yield F(ab')2 and Fc fragments. The digested proteins were separated using a UltiMate 3000 HPLC (Thermo Fisher Scientific) with a Kromasil 100–2.5-C4 column. Mass spectrometric analysis was performed on a QExactive orbitrap mass spectrometer (Thermo Fisher Scientific). Data were collected with Xcalibur software and analyzed with BioPharma Finder 2.0 (Thermo Fisher Scientific).

3. Results and discussion

In this report, retrovectors coding for GLP-1-Fc (Retro/GLP-1-Fc) were packaged in CHO-S cells and used to transduce CHO-S cells. Transduced CHO-S cells quickly recovered from transduction, and approximately 90% of the cells survived puromycin selection. Stable cell pools were evaluated in 14-day shaking flask fed-batch culture. GLP-1-Fc stable expression cell culture supernatants were analyzed by size-exclusion chromatography with dulaglutide as a reference standard and CHO-S culture supernatant as a negative control. No prominent peaks with retention time identical to that of dulaglutide were detected in the CHO-S supernatant (Background). GLP-1-Fc yields of stable cell pools were 0.3 to 0.7 g/L. Retrovectors from CHO-S were also used to transduce CHO-DG44, and stable cell pools had GLP-1-Fc productivities of 0.1–0.3 g/L. We also packaged retro/GLP-1-Fc in CHO DG44 cells for CHO DG44 cell transduction. GLP-1-Fc yields in CHO DG44 stable cell pools were 0.1 to 0.3 g/L.

To obtain single-cell clones with high yields, culture supernatants in 96-well plates were analyzed by SDS-PAGE and ELISA to determine productivities. Top 100 clones were scaled up to 15 mL and 50 mL TPP mini-bioreactors for further productivity analysis with ELISA. Finally, top 8 clones were evaluated by 14-day shaking flask fed-batch culture. The supernatants were analyzed by size-exclusion chromatography. These top 8 clones have yields of 1.0 to 2.1 g/L (Table 1). These clones were obtained within 45 days post retrovector transduction, which is significantly shorter than that of traditional cell line generation technology. To obtain higher productivity, single-cell clones No. 2 and No. 4 were transduced once more with Retro/GLP-1-Fc. Single-cell clones isolated from new stable cell pools have productivities of 2.65–3.15 g/L (Table 1). GLP-1-Fc stable expression CHO-S cells were also generated with retrovectors packaged in HEK-293. The productivities of top 10 clones are 0.8–2.0 g/L (Single transduction). We could not conclude which packaging system gave rise to relatively higher yielding CHO-S stable cell clones, as available data are limited. However, generation of CHO-S stable cells with retrovectors from HEK-293 is more difficult. Almost 90% of transduced cells died at 4 days post transduction. Except for retroviral transduction-induced cell stress, the following could be other reasons: 1. Change of culture medium during the transduction process; 2. Withdrawal of FBS after transduction. We also generated GLP-1-Fc stable expression CHO-S cells, with the pCHO1.0 plasmid (Thermo Fisher Scientific) bearing a GLP-1-Fc expression cassette. Productivities of top 10 clones are 0.8–1.6 g/L after two rounds of DHFR/MTX-mediated gene amplification. Most of these top clones generated by different methods exhibited a peak viable cell density (VCD) of 30–40 $\times$ 10^6 cells/mL, maintained high cell viability (>70%) throughout the 14-day fed-batch culture, and had a similar growth profile and metabolic profiles (lactate, glucose, and glutamine) in shaking flask fed-batch productions using the Dynamis media and Efficient Feed C+ supplement (data not shown). These parameters indicate that stable cells generated by this new method have similar cell growth, nutrient consumption, and metabolite production rates as those of stable cells generated by traditional methods. Thus, productivities of stable cells generated by different methods are comparable. The cell culture process could be further optimized based on these parameters to obtain higher productivity and suitable post-translational modifications of GLP-1-Fc.

Table 1
GLP-1-Fc recombinant protein productivities of single-cell clones. Top 8 single-cell clones generated from retrovector single transduction were fed-batch cultured in shaking flask and analyzed by size-exclusion chromatography (SEC). Single-cell clones No. 2 and No. 4 were transduced once more with retrovector. Top 11 clones were fed-batch cultured and analyzed by SEC. Clone No. 2/- and 4/- indicate single-cell clones originated from clone No. 2 and No. 4.

Single transduction		Duplicated transductions	
Clone No.	Titer (g/L)	Clone No.	Titer (g/L)
2	2.166	2/4	2.705
4	2.066	2/17	2.795
5	1.890	2/18	2.969
8	1.027	2/21	2.650
9	1.088	2/34	2.905
11	1.568	2/36	3.056
23	1.418	4/10	2.766
28	1.581	4/14	2.924
		4/16	2.849
		4/27	3.150
		4/31	2.910

For large-scale commercial manufacturing, cell lines routinely grow for over 25 generations to achieve high-density culture in larger bioreactors (working volume of up to 20,000 L). Long-term productivity stability is a key parameter for CHO stable cell line assessment. We passaged the master cell banks of 11 single-cell clones (duplicated transductions shown in Table 1) without selection pressure for 40 days in shaking flasks. CHO-S cells have a doubling time in the range of 17–20 h. Forty-day cultivation corresponds to approximately 48–56 generations. The single-cell clones at days 1 and 40 were fed-batch cultured in shaking flasks to determine GLP-1-Fc productivity. As shown in Fig. 1, the loss of productivity after 40 days of culture is less than 5%. Retrovectors could integrate multiple copies of viral DNA into transcriptionally active sites on cellular chromosomes. The insertion of the viral DNA into the host cell genome is catalyzed by the virus-encoded integrase. Murine leukemia virus (MLV) has distinct preferences, favoring integration at transcription start sites and CpG islands. Retrovectors could efficiently

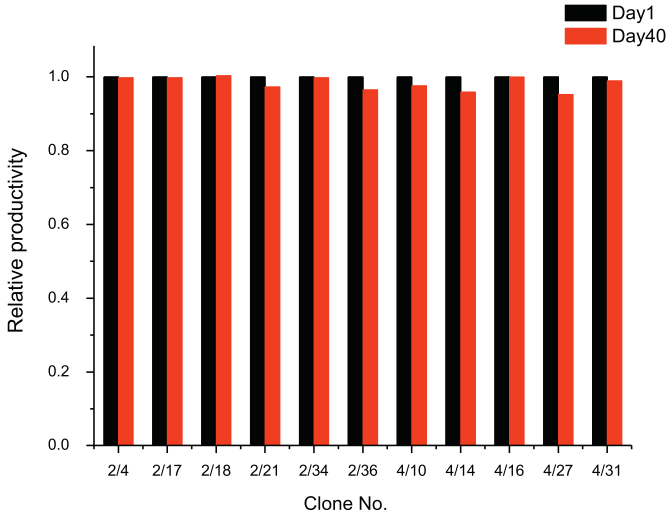

Fig. 1. Relative GLP-1-Fc productivities of 11 single-cell clones before and post 40 days of continuous culture. Master cell banks of 11 single-cell clones were continuously passaged without selection pressure for 40 days. Cell banks before (Day 1) and post 40 days of culture (Day 40) were evaluated in 14-day shaking flask fed-batch culture. GLP-1-Fc protein concentration was evaluated by size-exclusion chromatography (SEC). Productivities of cell banks on Day 1 were set to 1.0.

generate genetically stable, highly expressing cell lines [15,16]. This unique advantage of retrovectors has also been proven using the Catalent's commercial GPEx system [17].

For protein quality issues, the integrity of GLP-1-Fc was analyzed by peptide mapping. GLP-1-Fc recombinant proteins have identical amino acid sequence as that of dulaglutide molecules (data not shown). The bioactivity of GLP-1-Fc was evaluated by an *in vitro* fluorescence-based GPCR signal cascade activation assay. Compared with commercial Trulicity (relative bioactivity was set at 1.0), the relative bioactivity of GLP-1-Fc was 0.9–1.0.

Viral safety is a key concern in the development process of recombinant proteins from mammalian cells. Contamination events in the biopharmaceutical industry could be catastrophic. The main purpose of this new retrovector package system is to eliminate potential virus contamination at the cell line development stage. It is not applicable to clear viruses already existing in CHO cells, such as endogenous retrovirus-like particles (RVLPs), which could be detected by electron microscopy [18]. These RVLPs are not infectious, and they have not been associated with any disease state in humans. However, CHO stable cell lines generated with retrovectors packaged in HEK-293 cells have chances for viral contamination. CHO cells are permissive to multiple types of viruses such as Adenovirus, Parainfluenza virus, Bovine respiratory syncytial virus, Vesicular stomatitis virus, Reovirus, and Bluetongue virus [14]. All the viruses listed above could be transmitted from either HEK-293 cells [11,12,13] or fetal bovine serum [8]. We believe there are unknown or undetected viruses existing in HEK-293 and bovine serum. CHO cells may be permissive to these unknown or undetected viruses. Alternative retrovector packaging cell line, such as NIH 3T3, still faces a similar situation as that of HEK-293. It is urgent to eliminate potential virus contamination from the retrovector packaging system at the step of stable line generation. Our new packaging system will eliminate potential viral contamination from traditional packaging cell lines and fetal bovine serum.

4. Conclusions

Suspension CHO cells are relatively safe, clean, and reliable packaging cells for retrovectors. Generation of CHO stable cells with these retrovectors eliminates virus contamination from serum and traditional packaging cells such as HEK-293. Recombinant GLP-1-Fc proteins produced by this method have identical amino acid sequence and comparable bioactivity as those of commercial Trulicity. This feasible and reliable method should be applicable for other therapeutic protein-producing cell lines such as CHO-K1, CHO-GS, CHO-DXB11, NS0, and PER.C6.

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Conflict of interest

The authors declare that there is no conflict of interest.

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