Plasmid 71 (2014) 1–7

Contents lists available at ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Lentiviral vector platform for improved erythropoietin expression concomitant with shRNA mediated host cell elastase down regulation

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ARTICLE INFO

Article history:
Received 22 July 2013
Accepted 30 November 2013
Available online 8 December 2013
Communicated by Ananda Chakrabarty

Keywords:
Lentivirus vector
EPO
Elastase II
Inverse PCR
Metabolic engineering
shRNA delivery

ABSTRACT

Lentiviral vector (LV) mediated gene transfer holds great promise to develop stable cell lines for sustained transgene expression providing a valuable alternative to the conventional plasmid transfection based recombinant protein production methods. We report here making a third generation HIV-2 derived LV containing erythropoietin (EPO) gene expression cassette to generate a stable HEK293 cell line secreting EPO constitutively. A high producer cell clone was obtained by limiting dilution and was adapted to serum free medium. The suspension adapted cell clone stably produced milligram per liter quantities of EPO. Subsequent host metabolic engineering using lentiviral RNAi targeted to block an endogenous candidate protease elastase, identified through an *in silico* approach, resulted in appreciable augmentation of EPO expression above the original level. This study of LV based improved glycoprotein expression with host cell metabolic engineering for stable production of protein therapeutics thus exemplifies the versatility of LV and is of significant future biopharmaceutical importance.

1. Introduction

Achieving increased productivity of therapeutically important protein pharmaceuticals is a continuous process involving improvements in gene engineering, DNA delivery systems, host cell engineering and culture conditions (Baldi et al., 2005; Gustafsson et al., 2012; Ng et al., 2010; Peng et al., 2010; Wurm, 2004). Other than Chinese hamster ovary (CHO) cells, human embryonic kidney derived HEK293 cells are adaptable in serum free suspension culture and is thus an attractive platform for the transient or stable expression of recombinant proteins requiring proper post-translational modifications (Henry and Durocher, 2011; Loignon et al., 2008; Thomas and Smart, 2005; Walsh and Jefferis, 2006). Erythropoietin, the highly glycosylated 165 amino acids mature protein can be produced in mammalian host cell lines, including HEK293 cells, as recombinant human erythropoietin (rhEPO). Both the conventional preparations (epoetin α, epoetin β) and their hyperglycosylated analogue (darbepoetin α) is an accepted therapeutic agent for management of anemia due to chronic renal failure or several other underlying causes (Jelkmann, 2007). Global demand of EPO has remained very high and yield improvement with production ease therefore has remained a focused area as true for any therapeutically important recombinant product. One major problem in any recombinant protein production in heterologous hosts is the proteolytic degradation and resultant decrease in protein yield (Henry and Durocher, 2011; Warner, 1999). We report here obtaining a clonally derived HEK293 cells expressing rhEPO from the human EPO transgene delivered to the host cell through a HIV-2 derived LV

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http://dx.doi.org/10.1016/j.plasmid.2013.11.001

system developed earlier in our laboratory (Chande et al., 2013; Santhosh et al., 2008). Elastase II, one of the cellular proteases, was found to be secreted by HEK293 cells and the proteolytic enzyme showed significant reduction of rhEPO expression. Lentiviral delivery of shRNA to elastase II to the producer cell line showed an appreciable increase in rhEPO expression and the producer clone with down regulated elastase activity yields an amount of rhEPO in the laboratory scale flask culture not reported so far. This is the first report of harnessing LV for metabolic engineering of host cells for improved production of a therapeutically important recombinant protein.

2. Materials and methods

2.1. Cell culture

Mycoplasma free human embryonic kidney cell lines HEK293, (National Centre for Cell Science; NCCS, Pune, India) and HEK293FT (Invitrogen, USA) were maintained in DMEM supplemented with 10% FBS (Invitrogen) and 50 μg/ml Gentamycin (Nicolas Piramal, India) and K562 human erythroleukemia cells (NCCS) were cultured in IMDM supplemented identically, in a humidified incubator at 37 °C with 5% CO_2.

2.2. DNA extraction and gene cloning

Genomic DNA from cells was isolated by standard detergent lysis and phenol–chloroform extraction method and plasmids were extracted using QIAPrep plasmid DNA preparation kits following manufacturer's instructions (Qiagen, Germany). Most clones were first made in the T/A cloning vector pTZ57R (Fermentas, Lithuania), referred in the text as pTZ, further sub-cloned as required and all PCR primers referred are shown in Supplementary Table 1.

2.3. Transfection and lentiviral particle production

Typically 1×10^6 mycoplasma free 293FT cells were seeded in 60 mm Petri plate, incubated over night and transfected in fresh medium by either $CaCl_2$/BES method or using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. Transfection DNA mix consisting of transfer vector harboring the transgene and associated helper plasmids were used to prepare LV particles following multi-plasmid transfection procedure as described elsewhere (Chande et al., 2013).

2.4. Human EPO genomic clone

A 2.2 kb DNA fragment comprising start to stop codons of human EPO, devoid of 5′ and 3′ UTR, was PCR amplified using Expand long template PCR (Roche, Germany) from peripheral blood mononuclear cell genomic DNA of a healthy volunteer available in the laboratory and sub-cloned into the expression vector pcDNA 3.1 (Invitrogen).

2.5. cDNA synthesis and EPO expression

The genomic expression construct was transfected in HEK293 cells, cellular RNA extracted with Trizol (Invitrogen) after 48 h and cDNA was synthesized using RevertAid H-minus reverse transcriptase (Fermentas). The rhEPO cDNA was amplified using primers incorporated to have 6X His tag encoding nucleotides, sub-cloned in pcDNA 3.1, sequenced and aligned to the reference sequence in the database (GenBank accession no NM_000799). The missing nucleotides of the truncated EPO cDNA were incorporated by inverse long template PCR, sequenced and presence of complete reading frame was confirmed. The amplified fragment ends were polished with Mung Bean Nuclease (New England Biolabs, USA) and self-ligated with T4 DNA ligase (Fermentas). The reaction was transformed into the competent DH5αMCR *Escherichia coli* strain (Life Technologies, USA) and transformants were screened by PCR. The full length insert containing plasmid pcDNA-EPO was transfected in HEK293 cells and expression of recombinant rhEPO from the cell free culture supernatant was detected by immunoblotting and ELISA (StemCell Technologies, Canada).

2.6. Stable rhEPO production through LV transgenesis

A fragment encompassing the CMV promoter and rhEPO coding sequence was released from the pcDNA-EPO by BglII (polished)/NotI digestions and cloned at PmeI and NotI sites of pLV-*neo* (Chande et al., 2013) to obtain the transfer vector plasmid pLV-rhEPO. Viral particles were produced and target HEK293 cells were transduced (infected) with the virus in presence of 8 μg/ml polybrene (Sigma, USA). Cells were replaced with fresh medium after 16 h, incubated for 48 h and supplemented with 500 μg/ml G418 (Sigma) to select out transduced cells. The selection pressure was further increased to 800 μg/ml and rhEPO production by selected cells was checked by immunoblotting.

2.7. High producer clone selection by limiting dilution and adaptation to SFM

rhEPO expressing HEK293 cells were seeded into twenty 96 well flat bottom plates (Nunc, Denmark) at a concentration of 0.3 cell/well in antibiotic free medium. After 3 weeks, 351 emerging clones were selected and rhEPO expression profile from the culture supernatant of each well was analyzed by dot blot. Twenty-four clones showing appreciable protein levels by ELISA were selected and subsequently the highest producing clone was sorted out through three successive steps of culture expansion from equal cell seeding and EPO titer estimation. To adapt the clone in serum free medium (SFM), the adherent culture was trypsinized, washed and suspended directly into 90% SFM (ExCell HEK 293 SFM; JRH Biosciences, USA) supplemented with 10% DMEM (containing 10% FBS/gentamycin) in a hydrophobic surface coated T25 flask (Greiner, Germany). Culture was grown for a week, dead cells from the suspension culture were removed on Ficoll-Hypaque (Sigma) gradient and live cells were directly suspended