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Research Article

Transient Expression of a Recombinant Monoclonal Antibody in HEK293T Cells

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ABSTRACT

Background: Monoclonal antibodies (mAbs) are considered the most important and financially successful category of the biopharmaceuticals. Extensive optimization of the expression vector, host system and culture parameters are required for the successful production of active monoclonal antibodies in mammalian cells. In this regards, transient expression enables rapid and cost-effective production of recombinant proteins for initial characterization.
Methods: In the present study, an internal ribosome entry site (IRES) based bicistronic expression system has been evaluated for the transient expression of an anti-CD52 monoclonal antibody in mammalian cells. The IRES based bicistronic vector was generated through sequential cloning of the Light chain (LC), IRES, and Heavy chain (HC) in an intermediate vector and transfer of the resulting fragment to the expression vector. Transfection of the HEK293T cells was performed and antibody expression was analyzed in cell culture supernatant.
Results: Restriction enzyme analysis indicated successful cloning of the antibody coding unit in the expression vector. Analysis of EGFP expression indicated successful transfection of the HEK293T cells. Production levels of 220 µg/L of antibody were achieved in HEK293T cells during three days of culture.
Conclusion: Our results show the convenience and efficiency of the bicistronic expression system for transient expression of the whole monoclonal antibodies in mammalian cells.

Introduction

Until 2014, 44 therapeutic monoclonal antibodies were approved in Europe and USA while a larger number are being tested in different phases of clinical trials. The sales values of monoclonal antibodies in the market have peaked from 5.4 billion dollars in 2001 to 75 billion dollars in 2013. Most of this market goes to cancer treatment followed with and inflammatory and autoimmune diseases.[1,2] Mammalian cells appeared to be the most suitable expression system for complex biopharmaceuticals such as monoclonal antibodies. The unique characteristic of mammalian cells is their ability to generate human-like post-translational modifications such as glycosylation.[3]

Although recombinant antibody fragments appeared to constitute a promising category of biopharmaceuticals,[4] the market is currently dominated by whole monoclonal antibodies. Each antibody molecule is comprised of two heavy chains and two light chains. It has been reported that cells with higher expression level of the light chain compared to heavy chain are more capable of producing non-aggregated active antibodies.[5] Therefore the design of the expression vector has a very important role in the amount and quality of the produced protein.

So far different vector design strategies have been employed for the expression of monoclonal antibodies. The most common approaches include single gene vector expression system in which light chain and heavy chain coding genes are placed on two separate vectors,[5] dual gene vector system in which the two genes are transcribed from separate promoters placed on a single vector,[6] Bicistronic expression system in which light chain and heavy chain coding sequences are transcribed from a single promoter as a single transcript but they will be translated in two separate proteins due to the presence of an internal ribosome entry (IRES) element between the two genes,[7,8] and single ORF expression system in which a 2A-peptide coding sequence is placed between the two genes which are transcribed from a single promoter. In this system, the self-cleavage activity of the 2A-peptide facilitates separation of the two antibody chains after being transcribed.[9,10]

Studies have shown that the single gene vector system is the most ineffective strategy because the site and the

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number of genomic integration events in the host cell genome would be different for each vector.[5,6] Although the use of single vector dual gene strategy will facilitate co-integration of the two coding sequences, but putting a few promoters close to each other might result in promoter interference.[11] This problem has been solved in bicistronic and single ORF systems through the expression of the two genes from one promoter.[12]

The IRES sequences have been successfully employed for co-expression of multiple genes in mammalian cells as well as transgenic animals.[7,13] When placed between two genes, these elements facilitate cap-independent translation of the downstream genes while the upstream gene will be translated through the cap-dependent system. It has been shown that IRES mediated cap-independent translation occurs with lower efficiency compared to the original cap-dependent system.[14] Therefore, several reports have successfully used the light chain-IRES-heavy chain arrangement in which the ratio of the light chain expression would be higher compared to the heavy chain.[6,8]

CD52 is a 28 KDa GPI (glycosylphosphatidylinositol) anchored trans-membrane protein which is mainly expressed on lymphocytes and monocytes.[15] Anti-CD52 monoclonal antibodies have been extensively used as powerful immune-suppressive agents in stem cell transplantation, hematopoietic malignancies and auto-immune diseases.[16,17]

Transient expression systems offer several advantages including ease of development, speed and low cost for the expression of reasonable amount of recombinant proteins especially during early stages of the product development.[18] With the increasing number of therapeutic proteins in clinical analysis, optimization of transient expression systems has gained tremendous interest. Among different mammalian host cell lines CHO and HEK293T cells and their derivatives have been extensively employed for transient expression of complex recombinant proteins such as monoclonal antibodies. Therefore, in this study, we evaluated the feasibility of an IRES based bicistronic expression vector system for transient expression of an anti-CD52 monoclonal antibody as a model in HEK293T cells.

Materials and Methods

Vector Construction

To construct a bicitronic expression vector, the light chain (LC) coding sequence, IRES and heavy chain (HC) coding sequence were amplified and cloned sequentially in an intermediate vector with the final arrangement of the LC-IRES-HC, and the resulting fragment was sub-cloned in multiple cloning site of the pCMV-Basic expression vector (in house vector derived from pcDNA 3.1 hygro containing the CMV promoter and BGH poly A signal) using NheI and NotI restriction sites. To verify the cloning, restriction digestion was performed followed by sequencing. The in-house prepared pEGFP reporter vector was used as a control vector during the transfection studies.

Cell Culture

HEK293T cells (Pasteur Institute of Iran) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine serum albumin (FBS) (Biosera, France), 2 mM glutamine (Biosera, France), and 4500 mg/L glucose. Cultures were maintained in an incubator containing 5% CO2 at 37°C. The cells were passaged every 2-3 days at cell density of 0.2×10^6-0.3×10^6 cells/ml. Trypan blue exclusion method was used for estimation of cell concentration and viability.

Transfection and Transient Expression

24 hours prior to transfection, HEK293T cells were seeded in 24 well plates with the cell density of 0.1×10^6 cells/ml. Transfection was performed using the lipofectamine LTX reagent (Invitrogen, USA). A separate transfection was also performed with the pEGFP reporter vector to monitor the transfection efficiency. 72 hours after transfection, cell culture medium of transfected cells as well as control cells was collected and stored in -80°C until further analysis.

Antibody Expression Analysis

In order to analyze the amount of secreted antibody expression in the culture supernatant a human IgG1 Kappa chain specific ELISA was employed. One microgram of anti-IgG1 FC polyclonal antibody (Agrisera, Sweden) was coated in 4°C, overnight. After 3 times of washing with PBS buffer containing 0.05 tween 20 (Sigma, Germany), blocking was done in 200µl of 3% of skimmed milk (Sigma, Germany) for an hour. Following three more washing steps, 100µl of the culture supernatant and standard samples (0, 5, 10, 20 ng/ml) were added to each well and incubated for one hour. The wells were washed again and 100µl of the HRP-conjugated anti human kappa polyclonal antibody (Agrisera, Sweden) was added in 1/10000 dilution and incubated for one hour. After four washes, 100µl of the TMB substrate was added to each well and incubated for 15 minutes and reaction was stopped using 100µl of 1M HCL and the absorbance was read in 450 nm using a plate reader (BioTek, USA). A standard curve was created by plotting the absorbance of each standard sample against their concentration. The antibody concentration in each cell culture supernatant was then calculated based on the standard curve. Results were analyzed using student t-test and the differences between means were considered significant at $p< 0.05$.

Results

Vector Design and Construction

In this study a pcDNA 3.1 based vector which contains CMV promoter was used as the expression vector. The bicistronic cassette with the arrangement of light chain-IRES-heavy chain was cloned into this plasmid to obtain pCMV-mAb vector. The result of the enzyme digestion used to verify the cloning of the fragment is shown in Figure 1(a). Appearance of a 2800 bp band corresponding to the LC-IRES-HC confirmed successful cloning of the

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fragment. Figure 1(b) shows the final mAb expression vector map. Figure 1(c) shows the pEGFP reporter vector map.

Transient Transfection

Transient expression was performed during three days of culture. GFP expression was visualized in pEGFP transfected cells after 72 hours (Figure 2) which indicated successful transfection of the HEK293T cells.

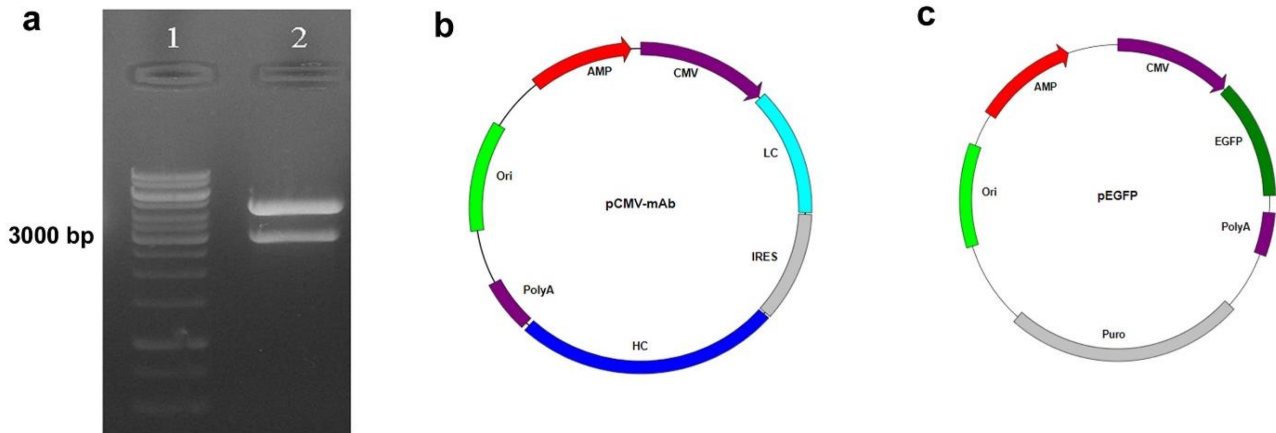

Figure 1. Restriction enzyme digestion and the final map for the pCMV-mAb expression vector. (a) Restriction enzyme digestion to confirm cloning of the LC-IRES-HC in NheI and NotI sites of the exporession vector. Lane 1: molecular size marker, and lane 2: digested plasmid. (b) The final map of the mAb expression vector. (c) The map of pEGFP reporter vector.

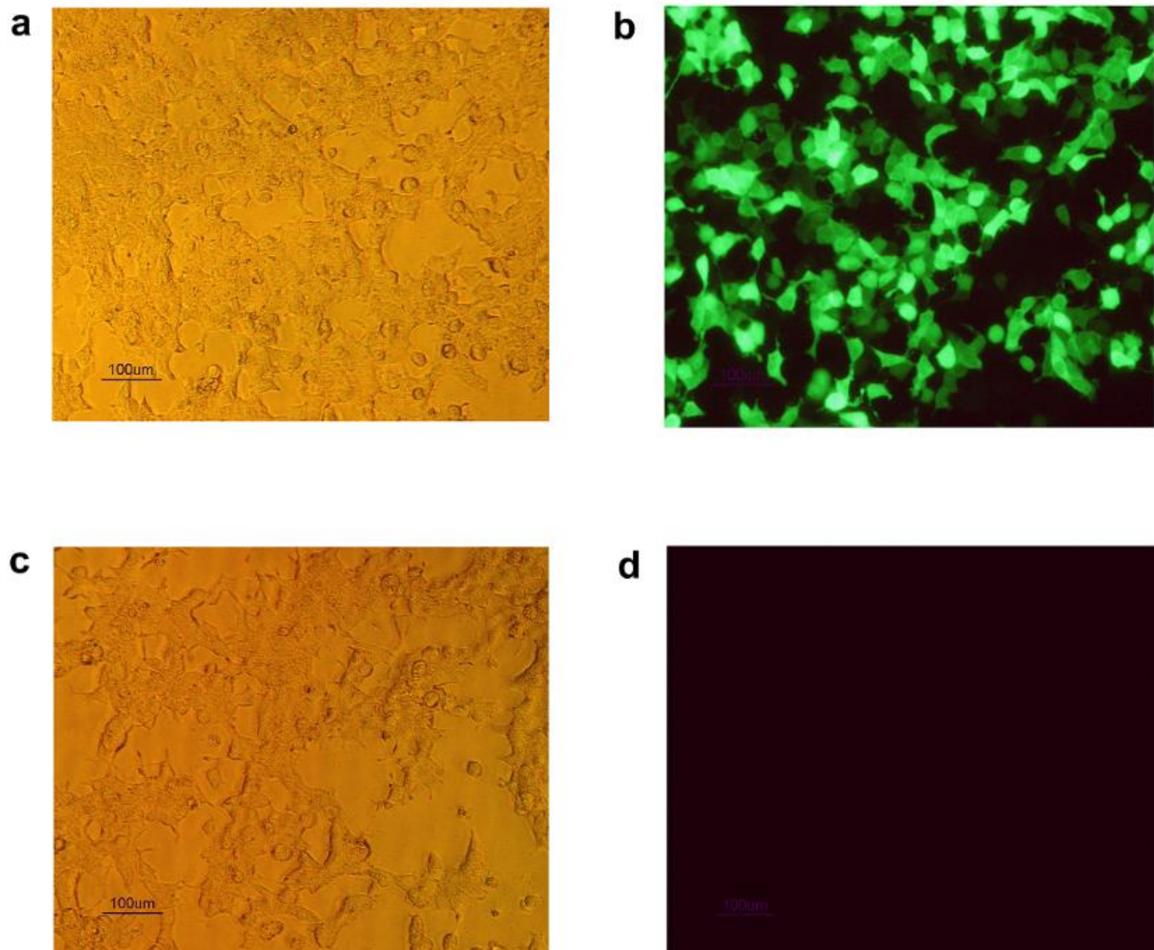

Figure 2. HEK293T cells were transfected with pEGFP reporter vector using lipofectamine LTX reagent and the efficiency of transfection was monitored after 72 hours using fluorescent microscopy. (a) Transfected cells under light microscopy. (b) Transfected cells under fluorescent microscopy. (c) Un-transfected cells under light microscopy. (d) Un-transfected cells under fluorescent microscopy.

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Expression Analysis

A human IgG1 specific sandwich ELISA was employed for expression analysis. As shown in Figure 3, antibody expression has been detected in culture of the transfected cells. The antibody concentration in HEK293T was estimated to be 220 µg/L. Supernatants of the un-transfected cells used as the negative control.

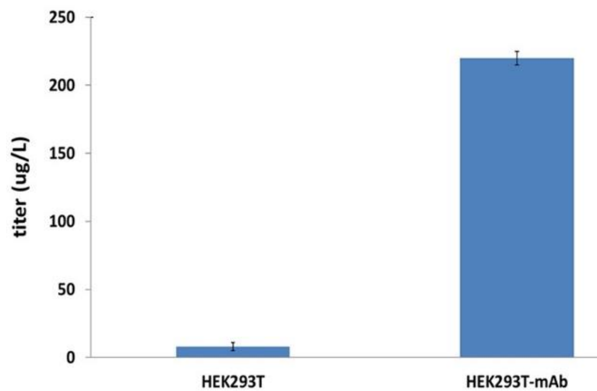

Figure 3. Analysis of antibody productivity of the transfected HEK293T cells in comparison to parental cells in transient expression. The presented values are the mean of three independent experiments and error bars indicate standard deviation.

Discussion

Although three decades have passed since the production of the first therapeutic recombinant protein in mammalian cells, development of the new strategies for the fast and efficient production of the complex heterologous proteins such as monoclonal antibodies still remains a challenge.[19-21] Different approaches such as optimization of the host system and expression vector as well as development of the novel screening technologies have been extensively used.[22,23] In this regard, transient expression has been known as a promising strategy for the fast production of a large quantity of proteins.[18] While stable expression of the recombinant therapeutics often requires several rounds of gene amplification which can take around six months, transient transfection assays can be performed in a several days to few weeks.[24,25] Among available host cells, CHO and HEK293 cells and their derivations have been widely employed for transient expression.[26]

Different groups have examined the efficacy of IRES based bicistronic or multicistronic expression systems for the expression of monoclonal antibodies in mammalian cells. For example, li et al.[6] showed successful transient antibody expression in CHO-KI and HEK293T cells using a bicistronic vector containing the antibody light chain and heavy chain. Ho et al.[8] also developed an effective tricistronic expression vector for simultaneous expression of the monoclonal antibody light and heavy chain and DHFR gene for transient and stable expression in CHO-DG44 cells.

Furthermore, it has been shown that improved expression of the light chain versus that of heavy chains is necessary for effective production of antibodies in transient and stable expression. In IRES based co-expression systems the upstream gene often shows higher expression level compared to the downstream unit. Therefore, to obtain optimal expression of the antibodies the light and heavy chain coding sequences are placed upstream and downstream of the IRES, respectively.[8] Although a number of IRES elements have been identified in viral and cellular systems, the encephalomyocarditis virus (EMCV) IRES element remained as the most widely used element for co-expression of transgenes in mammalian cells due to its translational efficiency and reproducibility.[27] Therefore, EMCV element was employed in the current study with the final arrangement of LC-IRES-HC.

Our study performed in the time frame of 72 hours after transfection and resulted in antibody expression titers up to 220 µg/L in the culture medium of adherent HEK293T cells. The observed expression level in HEK293T cells was expected because this cell line can be transfected very effectively. Also, the expression vector used in this work contains a CMV promoter and the adenovirus EIA gene constitutively expressed in HEK293T cells is known to activate CMV promoter.[26]

HEK293 cells and their derivatives have been extensively employed for transient expression of recombinant proteins. In a study conducted by Swiech et al,[28] up to 200 µg/L of recombinant factor VIII (rFVIII) was expressed in serum-free suspension HEK293 cell culture. In this study a bicistronic lentivirus vector was employed for the simultaneous expression of the human ß-domain deleted FVIII and the GFP reporter gene expression.

In a report by Li et al.[6] adherent HEK293T cells were employed for the expression of a monoclonal antibody using mono and bicistronic expression vectors. According to this study, up to 4 mg/L of antibody was expressed using the bicistronic system utilizing CMV promoter, which was significantly higher that the titers obtained from monocistronic dual vector system.

Vink et al.[29] developed an efficient episomal transient expression platform using fast growing suspension cell line, HEK293-F, using a dual vector expression system which resulted in antibody titers up to 400 mg/L during 4-6 days of culture.

Han et al.[30] employed a transient expression system based on suspension HEK293-F cells for the expression of osteopontin as a highly glycosylated protein. During this study 27 ng/L of recombinant protein was produced in shaker flask culture.

In a report by Jäger et al.[31] titers of 10-20 mg/L of recombinant scFv-Fc antibody were achieved in adherent HEK293T cells transfected with polyethyleneimide (PEI). Utilization of an EBNA based episomal system for HEK293-6E suspension cells in serum free culture improved the titers up to 10-fold to around 140 mg/L scFv-Fc antibody.

Based on these findings, it can be elucidated that a combination of factors including the nature of recombinant protein, structure of expression vector, culture mode (adherent vs suspension) and cell density can affect the productivity of HEK293 cells.

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Conclusion

The results of this research show the successful expression of an anti-CD52 model monoclonal antibody using a bicistronic expression platform. This design makes it possible to manufacture an acceptable amount of monoclonal antibodies in a short time frame. Application of this system in suspension adopted high density cell culture systems can certainly improve the production yield. Furthermore, conducting complementary research with regards of the antibody quality and binding properties can improve our perspective on the efficiency of this expression platform.

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Conflict of Interests

The authors claim that there is no conflict of interest.

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