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Research Article

Evaluation of the Effects of Human Beta-Interferon Scaffold Attachment Region (IFN-SAR) on Expression of Vascular Endothelial Growth Factor-Fc (VEGF-Fc) Fusion Protein Expression in Chinese Hamster Ovary (CHO) Cells

Ehsan Naghneh[1], Es'hagh Pourmaleki[2,3], Azam Rahimpour[2,3*]

[1]*Department of Genetics, East Tehran Branch, Islamic Azad University, Tehran, Iran.*
[2]*Nano-Technology and Tissue Engineering Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*
[3]*Department of Tissue Engineering and Applied Cell Sciences, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.*

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Abstract

Background: Recombinant anti-vascular endothelial growth factor (VEGF) monoclonal antibodies and Fc-fusion proteins have been widely used for the effective treatment of retinal neovascular diseases. In this regard, VEGFR-Fc fusions, which act as strong VEGF inhibitors, have been approved for the treatment of age-related macular degeneration (AMD) and diabetic macular edema (DME). Production of monoclonal antibodies and Fc-fusion proteins relies on mammalian host systems such as Chinese hamster ovary (CHO) cells. Application of genomic regulatory elements including scaffold/matrix attachment regions (SAR/MARs) can profoundly affect recombinant protein expression in CHO cells.

Methods: To construct the VEGFR-Fc expression vectors, the enhanced green fluorescent protein (EGFP) gene was replaced by the VEGFR-Fc coding sequence in pEGFP-SAR-puro and pEGFP-puro vectors. Recombinant plasmids were transfected to CHO-K1 cells using TurboFect transfection reagent. VEGFR-Fc expression was evaluated in transiently transfected cells as well as stable cell pools and clones using an enzyme-linked immunosorbent assay (ELISA).

Results: IFN-SAR showed no significant effect on transient expression of VEGFR-Fc during 72 h of culture. However, a 2.2-fold enhancement in VEGFR-Fc fusion protein titer was observed in IFN-SAR containing stable cell pools. Further evaluation of the VEGFR-Fc expression level in single-cell clones also indicated that clones with the highest VEGFR-Fc expression belonged to the pools transfected with IFN-SAR construct.

Conclusion: Our results indicate that the incorporation of IFN-SAR in expression vector can increase the expression of VEGFR-Fc in stable cell pools as well as single-cell clones. In contrast, transient expression of the fusion protein was not affected by IFN-SAR. More studies are needed to investigate the mechanism underlying this effect, including the analysis of mRNA expression and gene copy number in stable cell pools as well as clonal cells.

Introduction

Therapeutic monoclonal antibodies and Fc-fusion proteins are considered as one of the most promising and profitable classes of biopharmaceuticals due to their unique properties such as long half-life, high binding affinity, and the ability to recognize a wide range of antigens.[1,2] In addition, the possibility of generating the desired antibody fragments and fusion proteins through antibody engineering has further enhanced the interest towards these products.[3,4] Anti-angiogenesis therapy based on blocking of vascular endothelial growth factor (VEGF) or VEGF receptor (VEGFR) has been used as a therapeutic strategy to treat a wide range of human diseases including retinal diseases and cancers.[5,6] Soluble VEGF decoy receptors such as Aflibercept, which has been developed by fusion of the Fc region of human IgG1 antibody and some extracellular domains of human VEGFR, has been approved for the treatment of ocular diseases such as age-related macular degeneration (AMD) and diabetic macular edema (DME).[7,8]

Whole monoclonal antibodies, as well as Fc-fusion proteins, are mainly produced in mammalian expression systems such as Chinese hamster ovary (CHO), Sp2/0, and

*Corresponding Author: Azam Rahimpour, E-mail: rahimpour@sbmu.ac.ir

NS0 cells due to their ability in performing the appropriate post-translational modifications such as glycosylation.[9] CHO cells are considered as the main mammalian expression system for producing the recombinant therapeutic proteins.[10] However, mammalian cell-based expression systems suffer from major limitations, including low growth rate, low expression yield, and the need for expensive media. Several strategies have been employed for the improvement of recombinant protein expression in CHO cells including optimization of media, bioprocess development and expression vector engineering.[11]

Development of the optimized expression vectors is crucial for the high-level expression of mAb-based products in CHO cells. Accordingly, several epigenetic regulatory elements including insulators, scaffold/matrix attachment regions (SAR/MARs) and ubiquitous chromatin opening elements (UCOEs) have been successfully employed for improving the performance of mammalian expression vectors.[12-14]

SARs are DNA elements that are responsible for the attachment of chromatin to the nuclear matrix, which results in the generation of independent chromatin loops. Furthermore, SARs are involved in the regulation of transcription and chromatin accessibility.[15] Several studies have examined the performance of SAR-containing expression vectors for transgene expression in mammalian cells. In the current study, the effects of the beta-interferon scaffold attachment region (IFN-SAR) on the transient and stable expression of a VEGFR-Fc fusion protein were examined in CHO cells.

Materials and Methods

Plasmids and constructs

pEGFP-puro and pEGFP-SAR-puro vectors (Figure 1), which were constructed in previous studies (unpublished data), were used for the generation of VEGFR-Fc expression vectors. Briefly, pEGFP-puro was developed by ligation of a 1640 bp fragment containing CMV promoter, EGFP, and SV40 poly A from pEGFP-N1 vector (Takara Bio, Japan) to a 4060 bp fragment from pSilencer 2.1 U6 puro (Thermo Fisher Scientific, USA) that contained the origin of replication, ampicillin, and puromycin resistance genes. pEGFP-SAR-puro vector was generated by cloning a 2200 bp fragment containing human INF-SAR (kindly provided by Prof. Jurgen Bode, Germany) at the flanking sites of EGFP expression unit in pEGFP-puro vector using EcoRI and HindIII restriction site in reverse orientation. To construct the VEGFR-Fc fusion, first a 720 bp fragment containing human IgG1 Fc coding sequence was synthesized (Bioneer, Korea) and cloned in XhoI and NotI restriction sites of the pEGFP-puro and pEGFP-SAR-puro plasmids. Then, a 710 bp synthesized fragment encoding VEGFR domains (Bioneer, Korea) was cloned in KpnI restriction site, which allowed in-frame insertion of this sequence upstream of the Fc coding sequence to obtain the pFU-puro and pFU-SAR-puro expression vectors.

Cell culture

Adherent CHO-K1 (ATCC CCL-61) cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Biosera, France) at 37 °C in a humidified incubator with 5% CO_2. The trypan blue exclusion method was used to determine cell concentration and viability.

Transient expression

Plasmid transfection was performed using TurboFect transfection reagent (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. Briefly, 24 h before transfection, CHO-K1 cells were seeded at the density of 0.1×10^6 cells/well in 24-well plates. The following day, an equimolar amount of each expression vector (1000 ng of pFU-SAR-puro and 595 ng of pFU-puro) was diluted in 100 µl of serum-free DMEM-F12 medium and was mixed. Then, 2 µl of the TurboFect transfection reagent was added to the mixture, and incubated for 20 min at room temperature. The transfection reagent/DNA mixture was then added to each well and gently mixed. CHO-K1 cells were also transfected using the pEGFP-puro reporter vector to monitor the transfection efficiency. 48 h after transfection, pEGFP-puro transfected cells were analyzed using fluorescence microscopy (Nikon Instruments, USA), and flow cytometry (BD Bioscience, USA). VEGFR-Fc expression was also evaluated in the cell culture supernatant of pFU-SAR-puro and pFU-puro transfected cells using ELISA.

Development of stable cell pools

Stable pFU-puro and pFU-SAR-puro cell pools were generated by the transfection of CHO-K1 cells with the corresponding expression vectors in duplicates. 48 h post-transfection, the double transfectants of each vector were detached and then mixed. After that, the cells were diluted 1:10 in growth medium and seeded in 6-well plates. Also, the un-transfected CHO-K1 cells were seeded as negative control. Cells were then maintained in selective medium containing 5 µg/mL puromycin for 2 weeks. Puromycin selection was continued until colonies were formed in the transfected cells, and the un-transfected cells completely died. Puromycin resistant cells were cultured in puromycin containing medium until they reached >70% confluence. Cells were then expanded to T25 flasks for performing further analyses.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from pFU-puro and pFU-SAR-puro stable pools as well as un-transfected CHO-K1 cells using an RNA extraction kit (Favorgen, Taiwan). First-strand cDNA was synthesized from 5 µg of DNase-treated RNA using a first-strand cDNA synthesis kit (Vivantis, Malaysia). PCR was performed using gene-specific primers (Table 1).

Table 1. Primer sequences used in RT-PCR.

Primer name	Sequence
Fu For	GCCACCATGGTGTCTTACTG
Fu Rev	TAAGGATCCTCACTTGCC

Analysis of VEGFR-Fc expression

To estimate the VEGFR-Fc expression, the cells were seeded in 6-well plates at the cell density of 0.1×10^6 cells/ml, and culture supernatants were collected after 72 h. A direct enzyme-linked immunosorbent assay (ELISA) was employed in 96-well plates (Greiner, USA) to estimate the titer of the fusion protein in the cell culture supernatant. 1:2 and 1:4 dilutions of the cell culture supernatants were prepared in PBS. 100 µl of the dilutions were coated in each well in duplicates, and then were incubated at 37 °C for 1h. A human IgG1 antibody was used as the standard in 0, 6.25, 12.5, 25, 50, 100, and 200 ng/ml concentrations, and cell culture supernatants from the un-transfected CHO-K1 cells were used as negative control. The wells were then washed and blocked using 100 µl of blocking buffer containing 3% skimmed milk. After 1 h incubation and performing three washing steps, 100 µl of (HRP)–conjugated goat anti-human Fc (diluted 1:10000 in PBS buffer containing 1% BSA) was added to each well and incubated for another hour. Then, the wells were washed and 100 µl of tetramethylbenzidine (TMB) substrate (Sigma, Germany) was added to each well and incubated for 15 min. Finally, the reaction was stopped using 1M HCL and the optical density was measured at 450 nm. Specific productivity (Qp) was calculated using the following equation, where P is the titer (pg/ml), X is the cell density (cells/ml), and t is the culture duration in days.[16]

$$Qp = \frac{P2 - P1}{\frac{(X2+X1)}{2} \times (t2 - t1)}$$

Single-cell cloning

Single-cell cloning was performed in 96-well plates using serial dilution cloning.[17] The cells were diluted to 10 cells/ml density in DMEM/F12 medium, supplemented with 15% FBS and 2 mM L-glutamine, and then 100 µl of the cell suspensions were added to each well. After two weeks, single clones were identified under the phase-contrast microscope, and clones with detectable expression levels of VEGFR-Fc were identified using ELISA. 10 clones were selected from each pool and expanded in 24-well and 6-well plates.

Statistical analysis

Statistical analysis was performed using SPSS 18 software (SPSS Inc., USA). Student's t-test was used for comparing the means between two groups. P-value less than 0.001 ($P < 0.001$) was considered as statistically significant.

Results

Vector Construction

Cloning of the VEGFR-Fc fragment in pEGFP-puro and pEGFP-SAR-puro expression vectors was confirmed using restriction digestion analysis. Observing the specific bands at 1430 bp following restriction digestion indicated the presence of the VEGFR-Fc insert in the expression vectors (Figure 1a). Schematic representation of the VEGFR-Fc coding sequence (CDS) is shown in Figure 1c, and the maps of the resulting expression vectors are presented in Figure 1d-g.

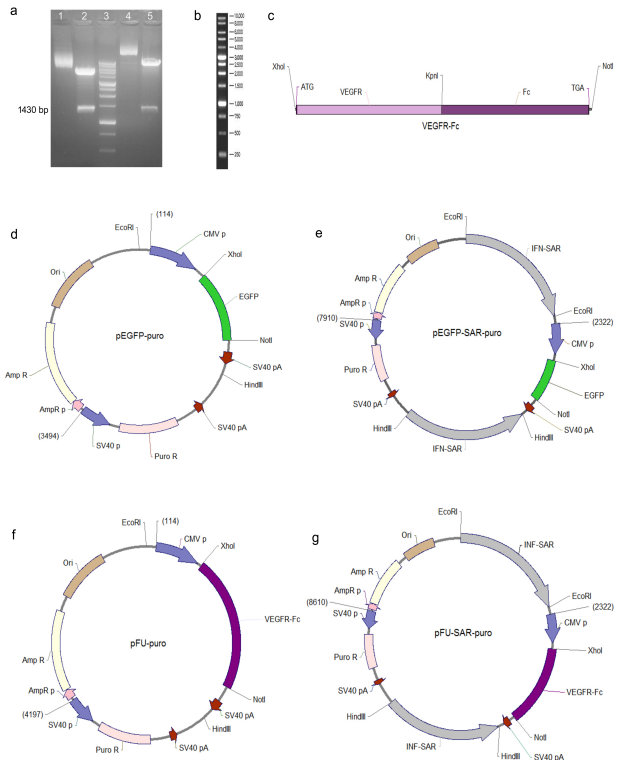

Figure 1. (a) restriction digestion analysis of recombinant pFU-puro and pFU-SAR-puro expression vectors. Lane 1: un-digested pFU-puro, lane 2: pFU-puro digested with XhoI and NotI. Lane 3: 1Kb size marker, Lane 4: un-digested pFU-SAR-puro, lane 5: pFU-SAR-puro digested with XhoI and NotI. The appearance of a 1730 bp band corresponding to VEGFR-Fc in digested vectors confirms the cloning. (b) DNA size marker. (c) schematic representation of the VEGFR-Fc CDS. (d) schematic map of pEGFP-puro vector, (e) schematic map of pEGFP-SAR-puro vector, (f) schematic map of pFU-puro vector, and (g) schematic map of pFU-SAR-puro vector.

Transient expression of the VEGFR-Fc

Transfection of CHO-K1 cells was performed using pEGFP-puro reporter plasmid as well as pFU-puro and pFU-SAR-puro expression vectors. Analyzing EGFP expression using fluorescent microscopy and flow cytometry indicated the successful transfection of the cells (Figure 2). No significant difference was observed in VEGFR-Fc expression in the cell culture supernatant of pFU-puro and pFU-SAR-puro transfected cells, indicating that the transient expression of VEGFR-Fc was not affected by the presence of IFN-SAR in the expression vector (Figure 3).

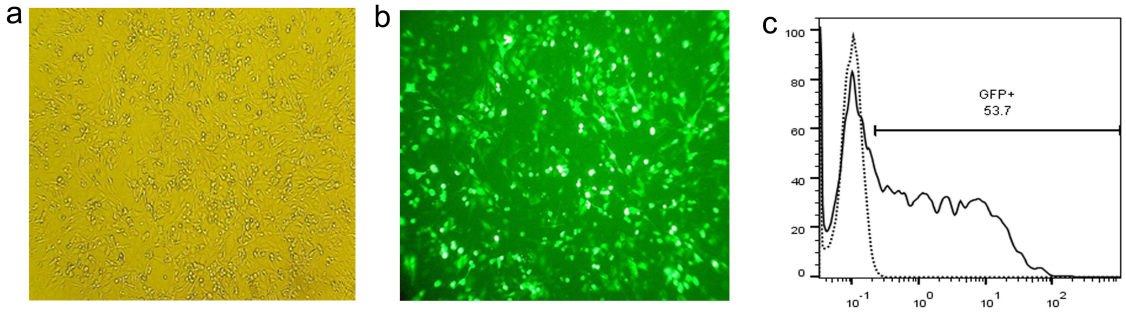

Figure 2. Analysis of pEGFP-puro transfected cells using fluorescent microscopy and flow cytometry. (a) light microscope image, (b) fluorescence microscope image, and (c) flow cytometry plot which shows the presence of 53% pEGFP positive cells.

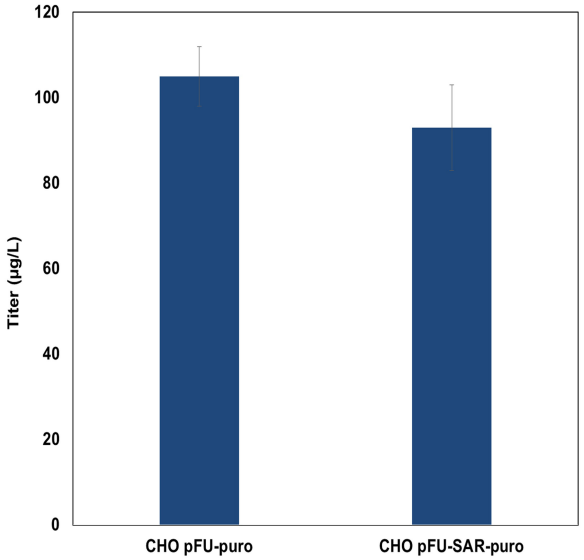

Figure 3. Transient expression of VEGFR-Fc in CHO-K1 cells. Expression analysis was performed 48 h post-transfection. The bars represent the mean values of three independent analyses.

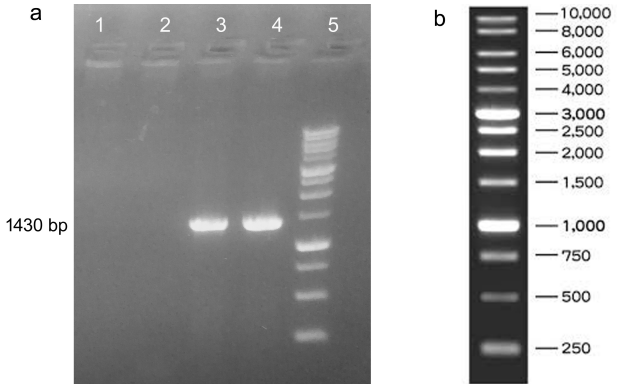

Figure 4. (a) RT-PCR analysis of VEGFR-Fc expression in pFU-puro and pFU-SAR-puro stable cell pools. Lane 1: negative control, lane 2: CHO-K1, lane 3: pFU-puro, and lane 4: pFU-SAR-puro. Observation of a 1430 bp band indicates the expression of the VEGFR-Fc mRNA in stable cell pools. (b) DNA size marker.

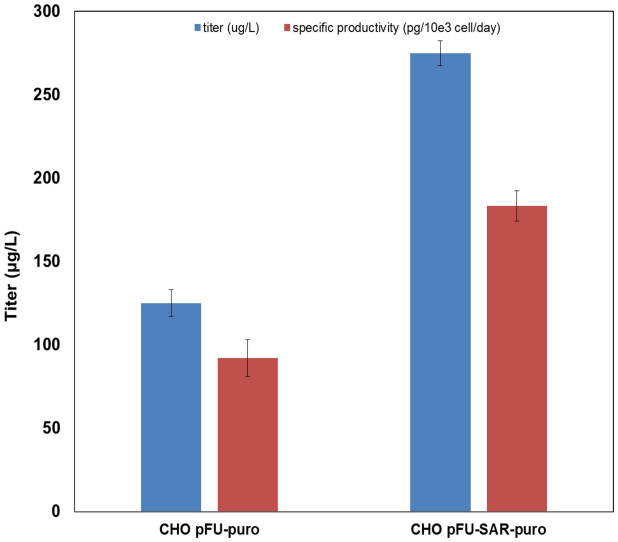

Figure 5. Analysis of VEGFR-Fc expression in pFU-puro and pFU-SAR-puro stable cell pools. Expression analysis was performed 72 h post culture. The bars represent the mean values of three independent experiments.

Evaluation of the VEGFR-Fc expression in stable cell pools

Expression of the VEGFR-Fc fusion protein in pFU-puro and pFU-SAR-puro stable cell pools was firstly evaluated using RT-PCR. As indicated in Figure 4, observing the specific bands at 1430 bp showed the successful expression of VEGFR-Fc in both pFU-puro and pFU-SAR-puro stable pools.

To evaluate VEGFR-Fc protein expression, CHO pFU-puro and pFU-SAR-puro stable cell pools were cultured at the cell density of 0.1×10^6 cells/ml. VEGFR-Fc titer in cell culture supernatant were measured after 72 h of cultivation, and the specific productivity was calculated. As indicated in Figure 5, compared to CHO pFU-puro, the titer and specific productivity of pFU-SAR-puro stable cell pool increased up to 2.2- and 2-fold, respectively ($P < 0.001$).

Evaluation of the VEGFR-Fc expression in single-cell clones

Analyzing the VEGFR-Fc titer and specific productivity in single-cell clones indicated improved expression of VEGFR-Fc in the clones derived from pFU-SAR-puro stable cell pool compared to the clones from CHO pFU-puro stable pool (Figure 6). SAR-C5 clone which was isolated from the pFU-SAR-puro pool showed the highest expression level with the titer and specific productivity of

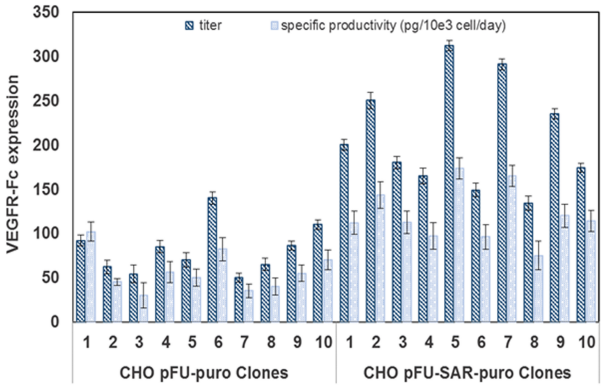

Figure 6. Evaluation of VEGFR-Fc expression in pFU-puro and pFU-SAR-puro derived clones. Expression analysis was performed 72 h post-culture. The bars indicate the mean values of three independent measurements.

312 ng/ml and 173 pg/10^3 cells/day, respectively, which were 2.2- and 2.1-fold higher compared to the best producing clone from pFU-puro stable pool ($P < 0.001$).

Discussion

SARs are genetic elements involved in chromatin organization and gene expression in higher eukaryotes.[15] Previous studies have suggested that SAR elements could increase the expression level as well as the stability of stable gene expression in CHO cells. As reported by Kim *et al.* (2004),[18] up to 7-fold enhancement was observed in the β-galactosidase reporter gene (B-GAL) expression from a plasmid-based expression vector using human β-globin MAR element. The authors did not observe any enhancement in transgene expression in transient expression.

In another study in 2005, Kim *et al.*[19] examined the expression of the B-GAL gene in a plasmid vector containing the human beta-interferon SAR element. Accordingly, a15- to 20-fold enhancement in B-GAL stable expression was reported in CHO DG44 cells; however, no positive effect was observed in the transient expression. Harraghy *et al.*[20] isolated a new SAR element from the mouse genome (S4 MAR). When incorporated into a monoclonal antibody expression vector, up to 2.7-fold increase was reported in cell-specific productivity compared to the control vector. SARs have been also employed for the improvement of the transgene expression from retrovirus vectors,[21] lentivirus vectors,[16] and transposons.[22]

In line with the previous studies, we evaluated the effects of INF-SAR element on the transient and stable expression of a VEGFR-Fc fusion protein in CHO-K1 cells. IFN-SAR element is one of the most studied SARs, which have been successfully employed in both plasmid-based and virus-based vectors.[16,23] Our findings from stable VEGFR-Fc expression in cell pools and clones were in agreement with the results of previous studies, with 2-fold enhancement in cell-specific productivity. The same effect was observed in the clonal cells derived from cell pools.

In our study, transient expression of VEGFR-Fc fusion protein was not affected by the presence of IFN-SAR in the expression vector. Generally, SARs are not considered as potent enhancers of transient gene expression, which is probably because SARs can affect the chromatin structure and organization after transgene integration into the genome. However, a few studies have shown some enhancements in the transient expression of the transgenes from SAR containing vectors.[24] Jia *et al.*[25] indicated that the position of DNA topoisomerase I gene (TOP1) SAR in the expression vector can affect transient and stable expression as well as transgene stability and ratio of positive colonies. These inconsistencies can be attributed to the differences in SAR elements, components of the expression vectors, and the transgenes among different studies.

Analyzing the VEGFR-Fc expression in single-cell clones derived from pFU-puro and pFU-SAR-puro pools also indicated substantial improvement in the fusion protein expression in the clones harboring IFN-SAR. Interestingly, we also observed notable variation among the pFU-SAR-puro derived clones. Although several studies have shown that the incorporation of the SARs in expression vectors can reduce the chromosomal position effect on the transgene, such effects may not be completely halted in the presence of SARs. Additionally, this variation may be also attributed to the differences in the transgene copy number. Finally, differences in protein expression and secretion machinery as well as metabolism among the clones can also affect the transgene expression.

Conclusion

In this study, the effects of the INF-SAR element on the transient and stable expression of VEGFR-Fc fusion protein were evaluated. Our findings show that IFN-SAR can significantly improve the expression of VEGFR-Fc in stable cell pools as well as clonal cells, while no effect was observed in the transient expression. Therefore, incorporation of the IFN-SAR in the expression vector can be considered as a promising strategy for effective expression of VEGFR-Fc in CHO-K1 cells. Evaluation of the expression stability of the clones and the quality attributes of the resulting fusion protein can further clarify the efficiency of this system.

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Conflict of Interest

The authors declare they have no conflict of interest.

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