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# A highly efficient modified human serum albumin signal peptide to secrete proteins in cells derived from different mammalian species

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ABSTRACT

Signal peptides (SPs) are key elements in the production of recombinant proteins; however, little information is available concerning different SP in mammalian cells other than CHO. In order to study the efficiency of different SPs to direct the traffic along the secretory pathway of the green fluorescence protein (GFP) and a scFv-Fc fusion protein; CHO-K1, HEK293 and NS0 cell lines were transfected in a transient and stable way. SP of human azurocidin (AZ), modified human albumin (mSA), modified *Cricetulus griseus* Ig kappa chain V III region MOPC 63 like (mIgκ C) and modified human Ig kappa chain V III region VG (mIgκ H) were evaluated. The efficiency of SPs to translocate a propeptide across the ER membrane was evaluated by fluorescence microscopy and flow cytometry for the GFP inside the secretory pathway, and by antigen-specific indirect ELISA for the scFv-Fc outside the cell. The mSA SP was successful in directing the secretion of the active proteins in these different types of mammalian cells, regardless of the transgene copy number. The goal of this work was to demonstrate that a modified version of SA SP might be used in different mammalian cells employing the same expression vector.



## 1. Introduction

Biopharmaceuticals represent preventive and therapeutic opportunities for a large number of human disorders. Today, about 250 recombinant therapeutic proteins have been approved and many more are in clinical trials [1]. Therefore, the study and development of innovative methods for the rapid production of recombinant proteins are essential [2]. These are mainly produced in mammalian cell lines to guarantee the quality of the post-translational modifications [3].

Chinese Hamster Ovary (CHO) cells are of particular interest for the industrial manufacturing of biopharmaceuticals. The main advantages of CHO cells are the feasibility of gene manipulation, the ability to create human-like glycosylation patterns, and the capacity for growth in high cell densities in serum-free suspension culture. In addition, the retina-derived primary human cells PER.C6, mouse myeloma-derived NS0, baby hamster kidney BHK-21 and human embryonic kidney HEK293 cells received regulatory approval for recombinant protein production [1,4].

Unfortunately, the cultivation of mammalian cells is very expensive and time consuming. In this regard, a variety of vectors and cell engineering strategies have been developed for generating high-producing mammalian cells [3,5–10]. Furthermore, protein secretion is known as one of the major bottlenecks in the productivity of mammalian cells. Several genes have been used to improve secretion from CHO cells, including molecular chaperones and mediators of secretory vesicle formation along the secretory pathway [11–15]. The translocation of secretory protein into the lumen of the endoplasmic reticulum (ER) constitutes the limiting step within the classical secretory pathway [16,17]. The protein secretion efficiency is not ensured by the natural signal peptide (SP) [18–20]. Even many prokaryotic and eukaryotic SPs are functionally interchangeable between different species [21–23]. Kober et al. [24] have selected 16 natural SPs from different species (mammals, fish, scorpions, snails, fungi, plants, viruses and bacteria) and evaluated the efficiency to increase the production of different recombinant proteins, including antibodies, in CHO-K1 cells. They demonstrated that azurocidin and serum albumin preprotein SPs from *Homo sapiens* improved product yield independently of the expressed protein. In this regard, there are reports of SPs evaluation in CHO cells but there is not enough information about these SPs in other mammalian cells [17–19,25]. In order to analyze the secretion efficiency of SPs and the productivity of the different cell lines, the

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expression of the green fluorescent protein (GFP) and a chimeric protein, based on a murine single-chain variable fragment (scFv) anti recombinant human interferon-α2b (rhIFN-α2b) fused with the fragment crystallizable domain of the human IgG1 (Fcγ1), was assessed.

## 2. Materials and methods

### 2.1. Cell lines and culture media

For CHO-K1, cell line growth and maintenance, Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (1:1) supplemented with 2 mM glutamine and 5% (v/v) fetal calf serum (FCS) was used. For HEK293 cell lines, DMEM supplemented with 2 mM glutamine 10% (v/v) FCS was used. For NS0 cell lines, DMEM supplemented with 2 mM glutamine and 20% (v/v) FCS was used. In all cases the pH was adjusted to 7.0. Media, FCS and consumables for cell culture were obtained from Gibco (USA), PAA (Argentina) and Greiner (Germany). Cell lines were cultured at 37 °C in a humidified incubator supplied with 5% $CO_2$ atmosphere.

### 2.2. Vector construct

The GFP and the chimeric protein scFv-Fc were used to analyze the traffic efficiency along the secretory pathway of different SPs, listed in Table 1 (AZ and highlighted SPs). Two different types of expression vectors were constructed. The first type of expression vectors were based on a lentiviral (LV) vector containing the GFP coding sequence (Fig. 1A) and the second type of expression vectors were based on a LV vector containing a scFv-Fc coding sequence (Fig. 1B). Both were under the control of Cytomegalovirus (CMV) promoter. The different signal sequences were synthesized by GeneArt™ Gene Synthesis (Thermo Fisher Scientific) and cloned between XbaI and XhoI restriction sites. The secretion efficiency of the modified SPs was checked *in silico* by the SignalP 4.1 bioinformatics prediction software [26]. All constructs were analyzed by DNA sequencing.

### 2.3. Transient and stable expression

For transient expression CHO-K1, HEK293 and NS0 cells were seeded in a 24-well plate ($1 \times 10^5$ cells per well) one day before transfection. Experiments were performed with the LipofectAMINE Reagent (Invitrogen) according to the supplier's instructions, using a mix of 1.5 μg of each vector and 1.5 μl of the LipofectAMINE Reagent. Intracellular fluorescence activity was measured on a Guava®EasyCyte™ cytometer (Guava Technology, USA) and analyzed on an Eclipse Ti-U Inverted fluorescence microscope (Nikon) 48 h after transfection. Extracellular scFv-Fc protein was measured by indirect enzyme-linked immunosorbent assay (ELISA).

48 h post-transduction, and in order to get stable expression, media were replaced with fresh growth medium containing 5 or 10 μg/ml puromycin (Sigma Aldrich, USA), depending on the cell line. Selective medium was changed every 3–4 days until control cells were dead. Stably-transfected cells were expanded for scFv-Fc quantitation.

### 2.4. Fluorescence measurements

Flow cytometry was performed on a Guava® Easy Cyte™ cytometer (Guava Technology, USA). This cytometer has a 488 nm blue laser for access to commonly used fluorescent dyes, and detectors to measure five different parameters (3 fluorescent channels and 2 light scatters). It allows simultaneous measurement of the side scatter (SSC), the forward scatter (FSC) and the green, yellow and red fluorescent emission which are collected by using the optical filters 525/30 nm, 583/26 nm and 690/50 nm, respectively. In addition, this equipment can measure samples from 96-well plates. Data acquisitions and analysis were performed using Guava CytoSoft™ 3.6.1 software. For each sample 5000 events were collected gating on the FSC vs SSC dot plot. Flow calibration and optical alignment was performed with the aid of Flow-Check Fluorospheres (Guava® Check kit, Hayward, CA, USA) before each determination. Cells were evaluated for the GFP signal (means fluorescence intensity multiplied by percentage of GFP positive cells) that is highly proportional to mRNA levels [27]. Data are representative of three independent experiments. The error bar represents the standard error of the mean (SEM).

### 2.5. scFv-Fc quantitation by indirect specific ELISA

Indirect ELISA was performed in 96-well plates (Greiner). Coating was achieved by incubating the plates with 50 ng per well of an rhIFN-α2b solution in 50 mM carbonate/bicarbonate buffer (pH 9.6) for 1 h at 37 °C and then ON at 4 °C. After blocking 1 h at 37 °C with 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS: 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 14 mM NaCl, 2.7 mM KCl, pH 7.4), plates were incubated with 2-fold serial dilutions of the chimeric protein standard (mentioned below) or the test samples for 1 h at 37 °C. Then, plates were incubated with an appropriately diluted polyclonal rabbit anti-human immunoglobulin (DAKO, Denmark) for 1 h at 37 °C. Finally, horseradish peroxidase (HRP)-labelled polyclonal goat anti-rabbit immunoglobulins (DAKO) diluted 1:2000 were added to the wells. After 1 h incubation, plates were incubated for 10 min with substrate solution (3 mg/ml *o*-phenylenediamine, 0.12% (v/v) $H_2O_2$ in 50 mM phosphate-citrate buffer). Absorbance was measured at 492 nm with a microtiter plate reader (Labsystems Multiskan MCC/340, Finland). Between every step, plates were washed 6 times with PBS containing 0.05% (v/v) Tween 20 (PBS-T). Dilutions of tested samples and antibodies were prepared in PBS-T containing 0.1% (w/v) BSA. Protein A affinity-purified scFv-Fc was used as standard to

**Table 1**
SPs and SignalP 4.1 scores.

| Signal peptide | Protein | GenBank accession number | Organism | Signal peptide sequence | GFP score | scFv-Fc score |
|---|---|---|---|---|---|---|
| AZ | Azurocidin preproprotein | NP_001691 | *Homo sapiens* | MTRLTVLALLAGLLASSRA | 0.842 | 0.868 |
| SA | Serum albumin preproprotein | NP_000468 | *Homo sapiens* | MKWVTFISLLFLFSSAYS | 0.844 | 0.875 |
| Modified SA | | | | MKWVTFISLLFLFSSSSRA | 0.873 | 0.890 |
| Igκ C | Ig kappa chain V-III region MOPC 63-like precursor | XP_003514704 | *Cricetulus griseus* | MGSAALLLWVLLLWVPGSNG | 0.937 | 0.942 |
| ModifiedIgk C | | | | MGSAALLLWVLLLWVPSSRA | 0.946 | 0.950 |
| Igκ H | Ig kappa chain V-III region VG precursor | P04433 | *Homo sapiens* | MEAPAQLLFLLLLWLPDTTG | 0.832 | 0.860 |
| ModifiedIgk H | | | | MEAPAQLLFLLLLWLPSSRA | 0.889 | 0.901 |

Underlined amino acids relate to the introduction of the last four codons from AZ SP 3′ sequence. The GFP and scFv-Fc scores depict the scores obtained after using the online server: SignalP 4.1 [26] to predict the presence and location of signal peptide cleavage sites in amino acid sequences from different organisms. Modified signal peptides scores are highlighted.

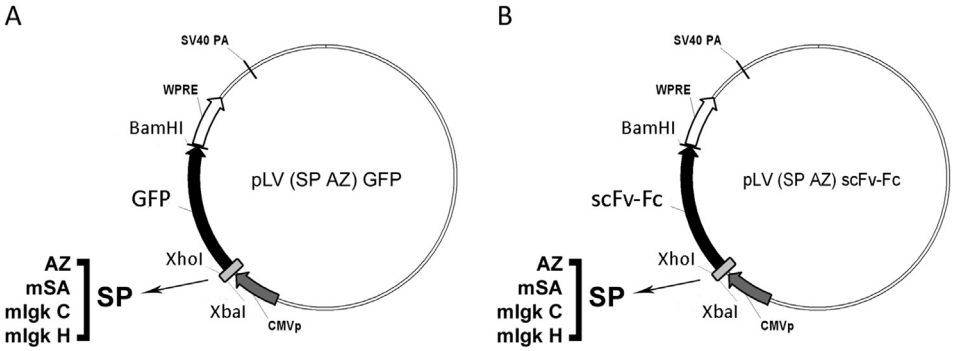



**Fig. 1.** Representation of different SP constructs. (A) GFP expression vector. (B) scFv-Fc expression vector.

prepare a 2-fold serial dilution curve from 1000 ng/ml to 7.8 ng/ml. The assay was reproduced in triplicate. Test samples were cell supernatants or cell extracts prepared at the same time of taking out supernatants. For that, after centrifuging the cell culture for 10 min at 800 rpm, supernatant was removed and the pellet was washed twice with PBS. Then cell extracts were prepared by 3 repeated cycles of freezing and thawing, and centrifuged at 4 °C for 10 min at 10,000 rpm.

### 2.6. Isolation of genomic DNA

A culture sample containing $5 \times 10^6$ cells was centrifuged at 800 rpm and after discarding the supernatant, cells were washed briefly with 1 ml ice-cold PBS and treated with lysis buffer (10 mM Tris-HCl, 20 mM EDTA, 200 mM NaCl, 0.2% (v/v) Triton X-100, 100 μg/ml proteinase) for 2 h at 37 °C. The extract was then treated with 100 μg/ml RNase for 1 h at 37 °C. Finally, the genomic DNA in the aqueous phase was precipitated with absolute ethanol and washed with 70% (v/v) ethanol. Dried genomic DNA was diluted with sterile water and stored at −20 °C before use. The concentration and purity of the genomic DNA was determined spectrophotometrically at 260/280 nm.

### 2.7. Real-time quantitative PCR

Quantitative PCR was carried out on a StepOne™ Real-Time PCR System (Applied Biosystems, USA). Reactions were performed in 20-μl final volume using 2X SYBR® Green PCR Master Mix (Life Technologies™) containing 100 nM of primers and $1 \times 10^3$ genome copies, calculated according to each genome size. The following LV vector specific primers to amplify the transgene were used: Fw LV 5′ CGCTGCTTTAATGCCTTTGT 3′ and Rev LV 5′ GGGCCACAACTCCTCATAAA 3'. Thermal cycling was initiated at 94 °C for 5 min, and the PCR process (94 °C 15 s, 60 °C 15 s, and 72 °C 15 s) was carried out for 40 cycles. Fluorescence was measured at 72 °C for 40 cycles. After PCR amplification, a melting curve was generated to verify the specificity and identify of PCR product. All samples were tested in triplicate. The standard curve was constructed by plotting the $C_T$ (threshold cycle) vs copy number of CHO-K1 cells genomic DNA containing a unique LV vector copy determined by genome sequencing.

## 3. Results and discussion

### 3.1. Evaluation of SP efficiency to target propeptides to the ER by transient transfection

The efficiency of a SP is determined by its ability to target a propeptide to the ER [28–30]. Therefore, the traffic across the secretory pathway was shown from GFP expression according Lippincott-Schwartz and Smith [31]. In order to evaluate the functionality of selected SPs, the presence and location of SP cleavage sites within the amino acid sequence of each propeptide were analyzed using the SignalP 4.1 server (Table 1). The last four codons of three SPs were changed by the corresponding AZ SP 3'codons. Modified SPs showed the same or even higher scores than wild type sequences. After this analysis, four vectors containing each SP followed by the GFP coding sequence (Fig. 1A) were constructed. Two independent transfection experiments using CHO-K1, HEK293 and NS0 cells lines were performed with these constructs. Fluorescence levels of cells transfected with GFP-containing vectors were evaluated by fluorescence microscopy (Fig. 2A) and measured by flow cytometry (Fig. 2B) 48 h after transfection. A SP-less construct (without SP) was used as expression control in cytoplasm. In consequence, the protein translation in the cytoplasm was a measurement of the highest GFP translation without the occurrence of the co-translational translocation limiting step. Considering only those cell lines transfected with plamids containing SPs, the GFP expression was higher when mSA SP was used as a signal sequence. Considering CHO-K1 cells, mSA SP showed fluorescence signals 5 to 7 times higher than AZ, mIgκ C and mIgκ H SPs. Also considering HEK293 cells, mSA SP fluorescence signals were 2–4 times higher, and for NS0 cells, they were more than 17 times higher than the same SPs. It is important to note that when using SP-constructs, lesser fluorescence signal within the cell was observed than when using a SP-less construct and it allowed concluding about the limiting step of the SPs-mediated secretory pathway. The fluorescence differences between SPs indicated the different efficiency of each SP, being the GFP signal highly proportional to mRNA levels [27].

Olczak and Olczak [32] observed that among the five heterologous SPs that they studied in directly transfected insect cells, AZ SP was the most potent one. Kober el al [24]. described the AZ SP also as a sequence to improve production rates. Our results, in the assay conditions herein described showed that other SPs are better than AZ. In this sense, it is important to consider that the C-ter region of SPs has been modified with the corresponding region of the AZ SP, which might modulate its activity.

Most cell culture medium contains fluorescent compounds such as phenol red that represents a false-positive reagent for the mentioned method. Therefore, as the final step of a protein secretion, the presence of another protein easier to be detected in the culture medium as the chimera scFv-Fc was evaluated. For this purpose, an antigen specific indirect ELISA for the scFv Fc constructs containing the same SPs employed for GFP was used.

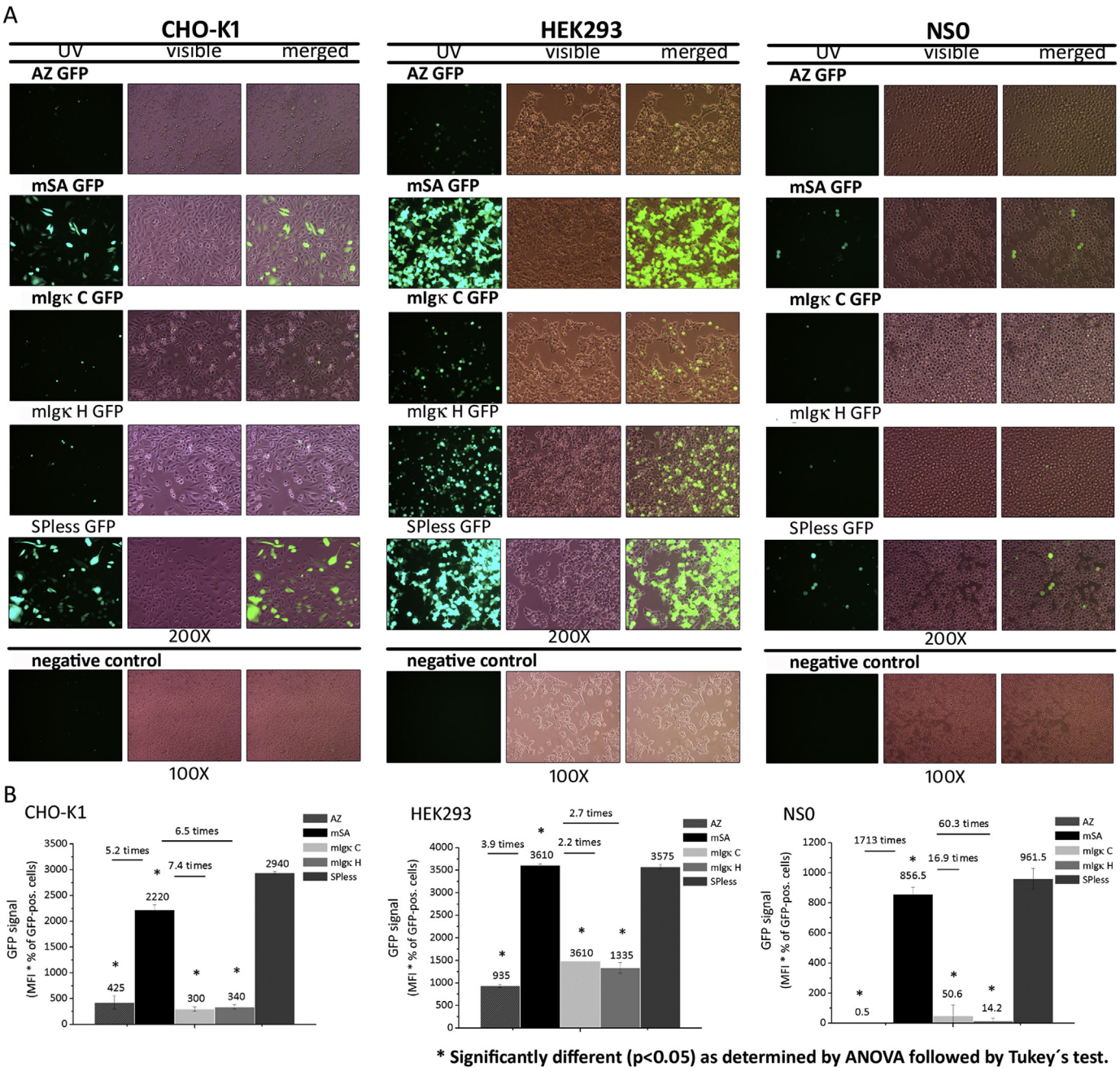



**Fig. 2.** GFP transient expression in CHO-K1, HEK293 and NS0 cells using different SPs. (A) fluorescence microscopy and (B) flow cytometry of cells 48 h after transfection. The upper horizontal lines compares the GFP expression using mSA SP respect to the GFP expression driven by the other SPs. MFI: mean fluorescence intensity. * Significantly different ($p < 0.05$) as determined by ANOVA followed by Tukey's test.

### *3.2. Evaluation of SP efficiency to direct proteins to culture medium by transient transfection*

In order to determine the efficiency of the selected SPs to export an antibody fragment fused to Fc region outside the cell, four vectors were constructed, each of them containing SP followed by the scFv-Fc coding sequence (Fig. 1B). CHO-K1, HEK293 and NS0 cells lines were transfected with these constructs. Two independent transfection experiments were performed for each cell line. Protein expression was measured on the 24 h supernatant culture media by indirect ELISA. Results are shown in Fig. 3. CHO-K1 cell line presented the same protein expression level for the distinct SPs assayed. Although the mIgκ H SP was the most effective in HEK293 cells, the mSA SP was almost as efficient as mIgκ H SP. Taking into account NS0 cells, the use of the mSA SP showed a double productivity when compared to other SPs. Only NS0 cells showed the same performance between experiments carried out using GFP or scFv-Fc. Thus, results agree with the literature in that amino acids downstream of the SP sequence can modify its efficiency [33–35]. Again, the lowest protein level in the supernatant culture was obtained with the AZ SP according to the GFP experiments.

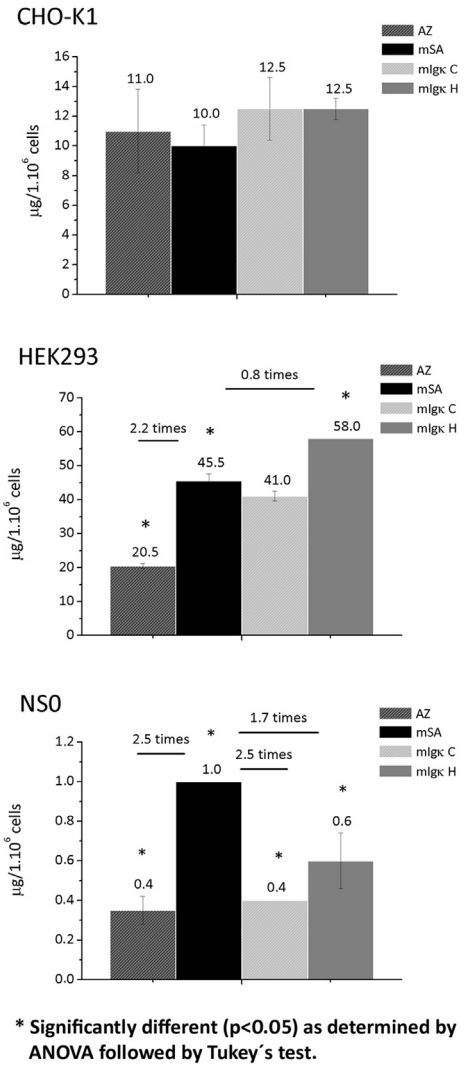



**Fig. 3.** Secreted scFv-Fc transient expression in CHO-K1, HEK293 and NS0 cells using different SPs measured by indirect ELISA. The upper horizontal lines compares the scFv-Fc productivity using mSA SP respect to the scFv-Fc productivity driven by the other SPs. * Significantly different ($p < 0.05$) as determined by ANOVA followed by Tukey's test.

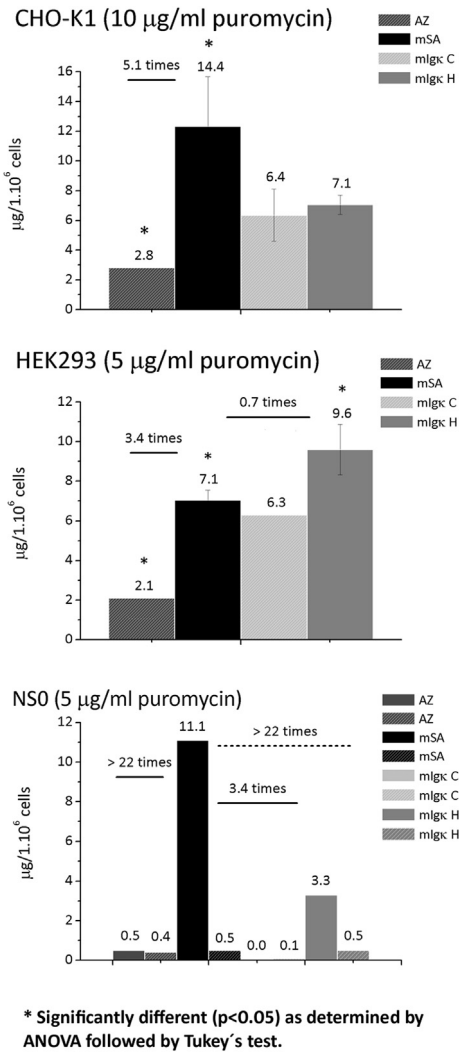



**Fig. 4.** Secreted scFv-Fc stable expression in CHO-K1, HEK293 and NS0 cells using different SPs measured by indirect ELISA. The upper horizontal lines compares the scFv-Fc productivity using mSA SP respect to the scFv-Fc productivity driven by the other SPs. * Significantly different ($p < 0.05$) as determined by ANOVA followed by Tukey's test.

### *3.3. Evaluation of SP efficiency to direct proteins to culture medium by stable transfection*

Kalwy et al. [36] reported that transient antibody expression levels and the productivity of the corresponding stable cell lines often did not correlate. In order to evaluate and compare the productivity of both types of transfections, CHO-K1, HEK293 and NS0 cells lines were transfected with scFv-Fc constructs. Two independent transfection experiments were performed for each cell line. With the aim of eliminating those remaining wild type cells, puromycin pressure at a final concentration of 5–10 µg/ml was performed. scFv-Fc expression was measured in the 24 h supernatant culture media by indirect ELISA. Results are shown in Fig. 4. The mSA SP was more effective in CHO-K1 and NS0 cells. Although the use of mIgκ H SP resulted in the highest expression of scFv-Fc protein in HEK293 cells, it was comparable to that obtained using mSA SP. In this way, a similar behavior in transient and stable transfection in HEK293 and NS0 cells was observed. With mSA SP, scFv-Fc specific productivity ranged from 2 to 5 times in CHO-K1, and it was more than 3 times higher in NS0 cells, in comparison with other SPs. The use of AZ SP produced the lowest scFv-Fc concentration under stable transfection condition. It is important to note that the productivity of NS0 cell line was shown for each replicate because values were different attending that the cell line is difficult to be transfected. Nevertheless, both replicates reflect that mSA SP increases the possibility to obtain the highest cell line productivity.

In the transient transfection the scFv-Fc expression levels were higher than in stable conditions. This difference could be due to factors that govern gene expression on both systems.

The functional scFv-Fc concentration in the secretion pathway was evaluated in cell extracts by indirect ELISA. This ELISA allowed determining the scFv-Fc concentration by its ability to bind rhIFN-α2b. For each cell type the ratio of the scFv-Fc that remains in the secretory pathway with respect to the totally-expressed chimeric protein was similar for the different SPs. However, NS0 cells retained more protein in the secretory pathway than other cell types, independently of the SP (Table 2). This evidence should be considered because the NS0 cells might increase the global time of

**Table 2**
scFv-Fc that remains in the secretory pathway (In) in comparison with the totally-expressed chimeric protein (In + Out).

| Cell line | scFv-Fc [In/(In + Out)] (%) | | | | |
|---|---|---|---|---|---|
| | AZ | mSA | mIgκ C | mIgκ H | Average |
| CHO-K1 | 8 | 6 | 8 | 5 | 6,8 ± 1,5 |
| HEK293 | 6 | 7 | 7 | 6 | 6,6 ± 1,1 |
| NS0 | 14/14 | 11/13 | – | 11/14 | 12,8 ± 1,3 |

In: inside the secretory pathway. Out: outside the cell.
(–) The scFv-Fc in the secretory pathway was undetectable using the corresponding SP.

production process of a recombinant protein, retaining functional protein or releasing it more slowly.

Although Olczak and Olczak [32] observed that in some cases the AZ SP can enhance secretion, but that the protein might be biologically inactive, the scFv-Fc produced in the present work was correctly folded using the different SPs because the protein was able to bind rhIFN-α2b.

### 3.4. Transgene copy number and relationship with secreted protein

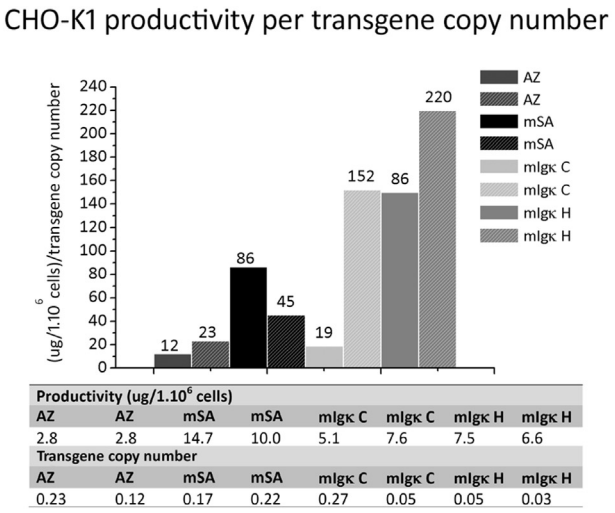
In order to evaluate the relationship between secreted protein and transgene copy number, a quantitative real time PCR was assessed (Fig. 5). The transgene copy number for each cell line was calculated by a standard curve using CHO-K1 cells genomic DNA containing a unique LV vector copy determined by genome sequencing. Differences in protein expression were observed between cell lines with a similar transgene copy number. The transgene copy number seems not to influence the secreted protein expression. Different cell lines transfected with the same construct and with similar transgene copy number, showed different expression levels, probably attributed to the genome transgene position [37]. It is important to note that HEK293 cells showed higher transgene copy number than CHO-K1 and NS0 cells. HEK293 have a good transfection capacity but this situation was not reflected as the best producer cell line. Inclusively, it showed the lower productivity per transgene copy number (relative productivity). This evidence should be evaluated in terms of transgene position taking into account the gene silencing mechanism or in terms of saturation in the expression machinery. Both situations might stress the cell and negatively influence the productivity.

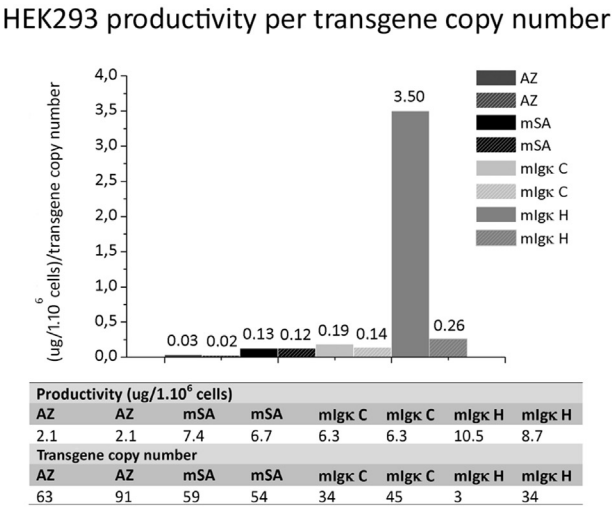
## 4. Conclusions

The relevance of this work is the demonstration that the same SP might be used to express proteins employing different types of mammalian cells. Particularly, mSA SP displayed the highest efficiency using CHO-K1, HEK293 and NS0 cells lines. For HEK293 cells, only the mIgκ H SP showed slightly better results correlating the scFv-Fc-transient expression with the stable-transfection, thus the mSA SP performance was only 0.8- or 0.7 times lesser than mIgκ H SP. Although HEK293 cells have a good transfection capacity according to the transgene copy number, this evidence has not resulted in an increased relative productivity.

NS0 cells retained a larger proportion of the recombinant protein in the secretory pathway than CHO-K1 and HEK293 cells. Therefore, this protein should be further evaluated in terms of protein folding, posttranslational modifications and also the time consumed during its transit inside the secretory pathway.

Finally, mSA SP has the ability of directing the protein secretion with a very high efficiency in cells derived from different mammals, leading to the production of recombinant proteins of commercial interest with expected properties of the selected cell line.



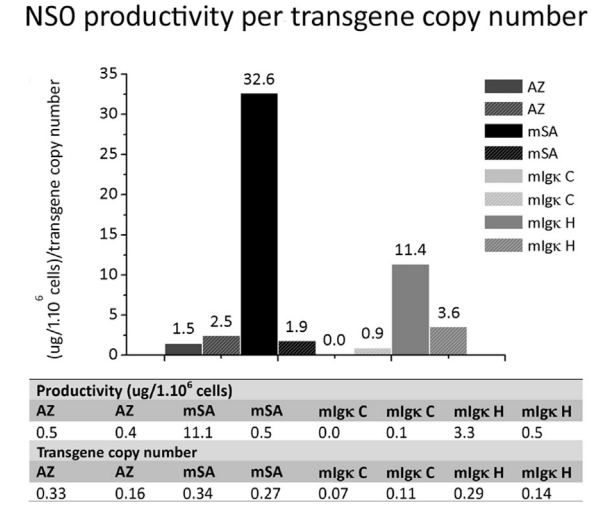




**Fig. 5.** Stable cell line productivity per transgene copy number determined by quantitative real time PCR. The replicates of each cell line are shown by different patterns. The cell line productivities and transgene copy number are shown at the end of each graph.

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