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Expression of Secreted Neutrophil Gelatinase-Associated Lipocalin in 293T Cell Using the Inducible Dual-Function System

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Abstract: Neutrophil gelatinase-associated lipocalin (NGAL) has emerged as a promising biomarker for the early prediction of acute kidney injury (AKI). The production of recombinant NGAL is considered to be necessary for the development of a detection method. This study intended to express the recombinant NGAL protein in 293T cell under the Tet-On inducible system and human serum albumin signal sequence (HSA-SS). The transfection efficiency and protein modulation were assessed by detecting the expression of the enhanced green fluorescent protein (EGFP) and secreted NGAL protein. Both proteins were detected only in the presence of a doxycycline (Dox) inducer. Cell toxicity was not found under any conditions. Moreover, a higher level of soluble NGAL protein in the supernatant secreted by HSA-SS compared with a native signal peptide (Nat-SS) was observed. In summary, this work successfully optimized the conditions for induction of NGAL expression. This system will provide as an efficient strategy to produce other recombinant proteins secreted from a mammalian cell.

Keywords: NGAL; Tet-On system; doxycycline; human serum albumin signal peptide; inducible vector

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1. Introduction

Acute kidney injury (AKI) involves a rapid decrease in the glomerular filtration rate, causing an accumulation of nitrogenous products in the kidney and blood. The high level of these products leads to kidney failure and a high mortality rate. Currently, the diagnosis of AKI is mostly based on an elevation in serum creatinine and blood urea nitrogen, but it seems likely that the creatinine level is a delayed response. As a marker, it is imprecise and lacks sensitivity and specificity [1], because it can only be detected after a loss of renal function beyond 50% [2,3]. Therefore, early predictive biomarkers of AKI are needed for diagnosis.

Neutrophil gelatinase-associated lipocalin (NGAL) is one of the most promising biomarkers for the prediction of acute renal impairment [4]. NGAL or lipocalin-2 (Lcn2) belongs to the lipocalin family of binding proteins. It is a secreted protein in the form of a monomer, homo-dimer, and hetero-dimer with the neutrophil gelatinase B enzyme [5]. An increase in the NGAL level in urine and serum has mainly been investigated in the development of AKI [6]. It can be rapidly detected in the blood and urine within a few hours of the onset of AKI [7]. This could be a potential early biomarker for clinical diagnosis.

The development of an immunological method and the evaluation of NGAL's biological functions requires a large amount of NGAL protein. The amount of purified protein ranging from a nanogram to a microgram per milliliter is need for using as a standard molecule in several detection methods such as an enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA) or chemiluminescent microparticle immunoassay (CMIA; Abbott Diagnostics, Abbott Park, IL, USA) [8]. Several expression systems have been established to produce recombinant human NGAL in various hosts, including prokaryotic systems [9,10] and eukaryotic systems [11]. The Chinese hamster ovary (CHO) cell line is the most widely used for the industrial manufacturing of biopharmaceuticals, due to their rapid growth, feasibility of gene manipulation, and the ability to culture them in serum-free medium at a high density [12]. However, CHO cells cannot produce some characteristics of human glycosylation but generate some glycans that are not found in human proteins, such as galactose-α-1,3-galactose (α-Gal) [13]. Human embryonic kidney 293T (293T) cells are also frequently used as host cells with the advantages of producing fully human post-translation modification [14], growing in suspension in serum-free culture, and expressing the high level of protein production.

The toxicity of recombinant proteins usually occurs through the accumulation of the overexpressed foreign proteins leading to cell apoptosis [15,16]. The development of several inducible expression vectors has been undertaken in order to modulate the recombinant protein expression at the desired time point. The tetracycline-inducible Tet-On/Tet-Off system has been broadly applied to regulate gene expression in eukaryotic cells. The Tet-On system comprises the reverse Tet transactivator (rtTA) fusion protein, which is a doxycycline-binding Tet-repressor mutant protein fused with the activator domain from the herpes simplex virus VP16 protein. This rtTA binds to the P_{tet} promoter and allows the activation of gene expression only in the presence of inducers such as tetracycline (Tet) or doxycycline (Dox), in a dose-dependent manner [17]. This system is valuable in applications in which gene expression must be sustained in the turn-off state for long periods, and gene induction (turn-on) can be manipulated under the control of the inducer [18].

There are many factors concerning types of host cell lines, cell culture medias, culture methods, transfection procedures, selection techniques, expression vectors, and the optimization of genes of interest for high-yield production of recombinant proteins [19]. Enhancing the transcriptional level using different approaches are considered such as the integration of a strong promoter sequence, an activator, an enhancer, and the manipulation of target gene by genetic engineering [20,21]. However, the expression of a protein does not always relate to its mRNA levels [22], and a rate-limiting step can be the secretory machinery. The proper signal peptide is a key factor involved in the precise translocation and secretion of proteins [23]. Regarding recombinant NGAL production, the human serum albumin (HSA) signal peptide has been reported as the most efficient machinery among others such as the native signal peptide of NGAL, the signal peptides of human interleukin-2 (IL2), gaussia luciferase (Gluc), and hidden Markov model-generated signal sequence (HMM38) [24].

In this respect, we applied the Tet-On inducible system and the appropriate signal peptide for the production of soluble NGAL protein in 293T cells. This system is able to both regulate the protein expression and enhance protein secretion for increasing the production yield. This feature of the vector design could be a model to further produce recombinant NGAL at a large scale.

2. Materials and Methods

2.1. Design and Construction of NGAL-Expressing Tet-On Vectors

Two tandem sequences of insert fragments, Nat/NGAL-IRES-EGFP and HSA/NGAL-IRES-EGFP, were designed. The complete coding sequence of human NGAL (NM_005564.4) with native (Nat) or human serum albumin (HSA) signal sequences (SS) at the 5′ end were linked with cellular internal ribosomal entry site (IRES) sequences followed by an

EGFP sequence. These insert fragments were separately synthesized into pcDNA3.1 vector by Genscript Biotechnology (Genscript Biotechnology, Piscataway, NJ, USA). For the construction of the inducible NGAL-expressing vector, the pLVX-TetOne-Puro vector (Clonetech, PaloAlto, CA, USA) containing the Tet-On inducible system was used as an acceptor vector. Two insert fragments were amplified using the Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). PCR products were double-digested with *Eco*RI and *Bam*HI, and then ligated with T4 DNA ligase (Thermo Fisher Scientific) into a linearized pLVX-TetOne-Puro vector digested with the same enzymes. Ligation products were transformed into XL-1 blue, resulting in two vectors termed pLVX-Tet-On-Nat/NGAL and pLVX-Tet-On-HSA/NGAL (Figure 1).

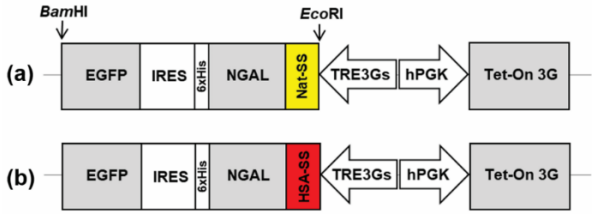

Figure 1. Schematic diagram of the bicistronic Tet-On inducible vector for NGAL and EGFP expression. The NGAL-6xHis tagged and EGFP genes were linked in tandem with IRES. The pLVX-TetOne-Puro vector was used as the acceptor vector. (**a**) The native signal sequence (Nat-SS) and (**b**) the human serum albumin signal sequence (HSA-SS) were separately inserted into the upstream of the NGAL gene, resulting in two plasmids named pLVX-Tet-On-Nat/NGAL and pLVX-Tet-On-HSA/NGAL, respectively.

2.2. Cell Transfection and Supernatant Collection

Transient transfection was performed by using the jetPRIME™ transfection reagent (Polyplus, New York, NY, USA), according to the manufacturer's instruction. Briefly, 2.5×10^5 cells/well of 293T cells were seeded into a 24-well culture plate one day before transfection. Two constructed plasmids were separately mixed with 50 µL of the buffer and 1 µL of jetPRIME™ transfection reagent, and incubated at room temperature for 10 min to allow complex formation. The mixtures were separately added to the 293T cells, resulting in NGAL-expressing cell named 293T-Tet-On-Nat/NGAL and 293T-Tet-On-HSA/NGAL. The culture media was changed 4 h after transfection. After 24 h, freshly prepared completed DMEM medium (Gibco, Paisley, UK) containing 1 µg/mL of DOX was added, and then, half of the media was replaced every two days, following the optimum condition from previous protocol [25]. EGFP expression was captured under a fluorescence microscope (Optika Microscopes, Bergamo, Italy). The culture supernatants were collected on day 6 after transfection to detect the presence of secreted NGAL. At the end of the experiment, transfected cells were collected for further experiments.

2.3. Cell Viability

Trypan blue exclusion assay was used to assess the cell viability and toxicity. Ten µL of cell samples were mixed with an equal amount of 0.4% trypan blue solution (Gibco, Paisley, UK) and measured under a microscope using a dual-chamber hemocytometer. The percentage of viable cells was calculated, and the mean ± SD of three independent wells was used for analysis.

2.4. Evaluation of EGFP Expression by Flow Cytometry

The cells samples were harvested and washed with DPBS three times. Cell pellets were re-suspended with DPBS containing 1% formaldehyde and analyzed using a Guava EasyCyte Plus Flow Cytometer (Merck Millipore, Billerica, MA, USA). Data were analyzed by FlowJo software.

2.5. Detection of Soluble NGAL by Western Immunoblotting

To analyze the molecular size and to assess the presence of NGAL in the supernatant, the supernatants of all conditions were separated by using 12% SDS-PAGE under reducing conditions, and further transferred to a PVDF membrane (GE Healthcare, Little Chalfont, UK). The membrane was blocked with blocking buffer (2% BSA in PBS) and probed with anti-His tag monoclonal antibody, followed by HRP-conjugated goat anti-mouse immunoglobulins. The reactive bands were visualized using the TMB membrane peroxidase substrate system (KPL, Gaithersburg, MD, USA).

2.6. Determination of NGAL Concentration

Cell pellets of all conditions were lysed on ice in RIPA buffer (Thermo Fisher Scientific) for 15 min. After incubation, samples were centrifuged at 16,000× *g* for 10 min at 4 °C and the cell lysate supernatants were collected. All culture supernatants and cell lysates were measured by the Human Lipocalin-2/NGAL DuoSet ELISA kit (R&D, Minneapolis, MN, USA), according to the manufacturer's protocol. Briefly, a 96-well microtiter plate was coated with 2 µg/mL of rat anti-human lipocalin-2 capture antibody and incubated overnight at room temperature. Then, the blocking solution (1% BSA in PBS) was added after the washing step and incubated at RT for 1 h. After incubation, the culture supernatants, cell lysates, and recombinant NGAL standards were added to the coated wells and incubated at RT for 2 h. After the washing step, anti-human lipocalin-2 capture antibody-mediated NGAL target was detected using 25 ng/mL biotinylated goat anti-human lipocalin-2 detection antibody. After 2 h of incubation at RT, the antibody-NGAL sandwich complex was monitored by streptavidin conjugated to horseradish peroxidase (HRP). The optical density of the color-forming TMB substrate was measured at 450 nm by a microplate reader (BioRad, Hercules, CA, USA). The concentration of NGAL of each sample was calculated from the standard curve.

2.7. Statistical Analysis

The results were expressed as the mean ± SD of three independent experiments. Statistical significance was determined using Student's t-test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Construction of a Dox-Inducible Vector for Dual Gene Expression

Two genes, NGAL and EGFP, were cloned into pLVX-TetOne-Puro vectors harboring the TRE3Gs promoter (Figure 1). TRE3Gs is an inducible promoter that is activated by doxycycline or tetracycline. It is an advanced Tet-responsive element (TRE)-containing promoter, which has been modified in terms of the sequence to minimize background expression while maintaining high levels of induced expression [26]. The TRE3Gs promoter lacks a binding site for endogenous mammalian transcription factors, and the minimal CMV promoter is inactive in the absence of a doxycycline transactivator protein, resulting in the low level of basal expression. The Tet-On 3G transactivator protein is expressed constitutively by the human phosphoglycerate kinase 1 (hPGK) promoter. In the presence of Dox inducer, the Tet-On 3G transactivator protein binds to Dox and undergoes a conformational change, leading to the recognition with the TRE3Gs promoter and the activation of the NGAL and EGFP expression.

3.2. Dox-Induced EGFP Expression in Transfected 293T Cells

To investigate the efficacy of Dox-inducible system, EGFP expression was continually monitored until day 6 after Dox treatment. The EGFP expression was observed in cells harboring constructed plasmids but not in the 293T control. The leakage expression was observed in non-induced condition, as shown by the very low signal of EGFP. Therefore, the bright fluorescence signal was detected in Dox-induced condition. Moreover, a higher fluorescence level of EGFP was observed in 293T-Tet-On-HSA/NGAL compared to 293T-Tet-On-Nat/NGAL (Figure 2a). These results demonstrated the success of transfection and regulation of protein expression by Dox inducer. The percentage of cell viability of all conditions was not significantly different, as shown in Figure 2b. This result indicated that the expression of transgenes was associated with low toxicity, and it did not affect the host cell viability.

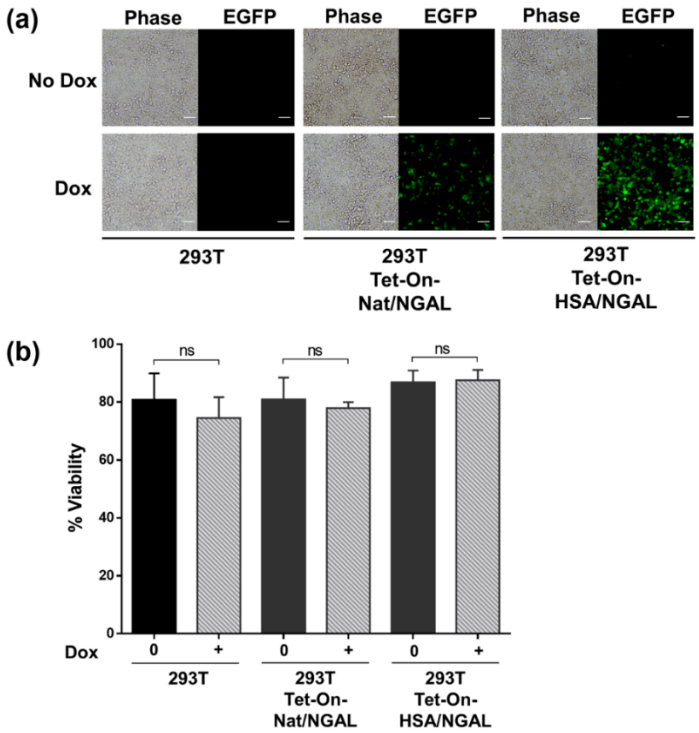

Figure 2. Expression of EGFP and cell viability after Dox induction. (**a**) The EGFP expression was observed under fluorescence microscopy at 20× magnification (Scale bar: 100 μm). (**b**) Cell viability was evaluated by the trypan blue exclusion method. Results were obtained from triplicate experiments (mean ± SD). ns indicated non-significant data at $p < 0.05$.

3.3. Level of EGFP Expression in Transfected Cells

To measure the effect of the Dox-induced condition on the level of EGFP expression, cells were collected at day 6 post-induction and determined by flow cytometer. The results revealed significantly different in the percentage of EGFP-positive cells and the mean fluorescence intensity (MFI) between Dox-induced and non-induced conditions in both transfected cells (Figure 3a,b). Therefore, comparing two determined parameters in both cells were not significantly different. This result demonstrated the same level of transfection and protein induction efficiency by Dox inducer in both cells.

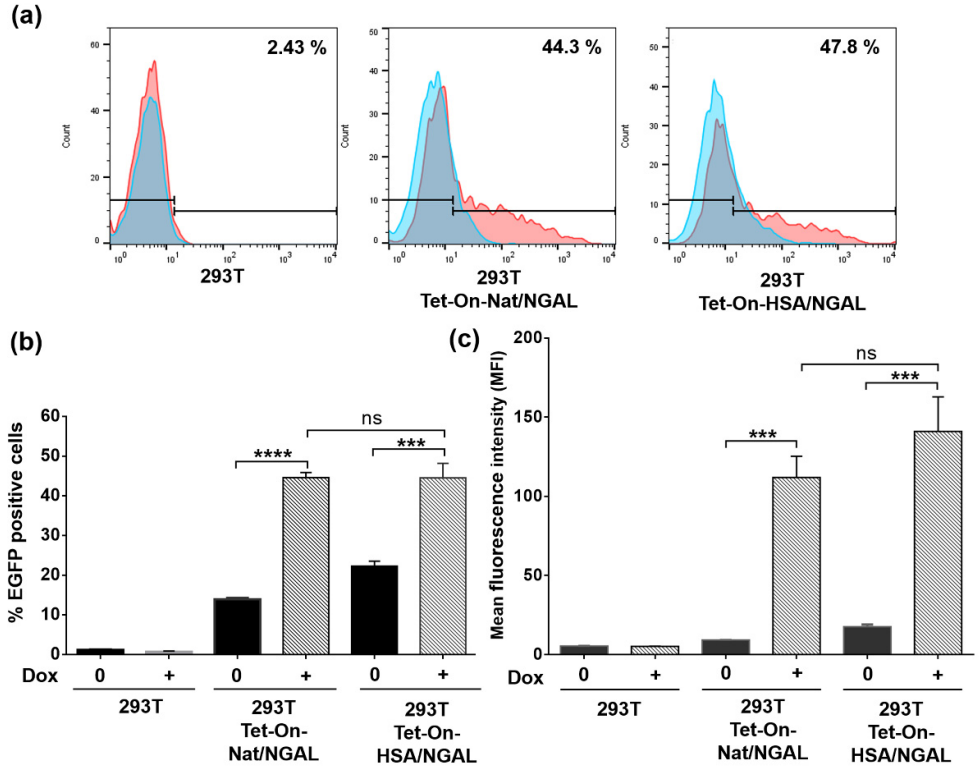

Figure 3. Flow cytometry analysis of EGFP-positive cells. (**a**) Representative overlay histograms showed the percentage of cells expressing EGFP. The blue and red peaks represented non-induced and Dox-induced conditions, respectively. (**b**) Percentage of EGFP positive cells and (**c**) MFI value were analyzed by FlowJo software. The results represented by triplicate experiments (mean ± SD). Data were analyzed using Student's *t*-test (*** $p < 0.001$, **** $p < 0.0001$).

3.4. Quantification of Secreted NGAL in the Supernatant

Culture supernatants of all conditions were examined by Western blot analysis using anti-6xHis tagged antibody. The results revealed that the reactive band at approximately 22 kDa, which represented the molecular size of NGAL, was observed in both Tet-On–HSA-SS and Tet-On-Nat-SS induced cells with different intensities (Figure 4a). In addition, there was no band representing the degraded NGAL, and any band was observed under non-induction condition. The result of NGAL quantification by ELISA showed that the level of the secreted NGAL protein under Dox-induced condition was significantly higher than non-induction conditions (Figure 4b) related to the intensity of reactive bands. Interestingly, the level of NGAL expression by HSA-SS was significantly higher than Nat-SS (Figure 4b), which indicated that HSA-SS promotes the high level of secreted NGAL protein in culture supernatant. These results could be summarized that the optimization of conditions for the induction of NGAL expression required both Tet-On regulation system and HSA signal peptide.

The intracellular level of NGAL was also evaluated by ELISA (Figure 4c). The same level of NGAL concentration in cells harboring both expression plasmids was observed. This result indicated an equal level of intracellular NGAL accumulation and also supported the efficiency of HSA-SS on the secretion of NGAL extracellularly.

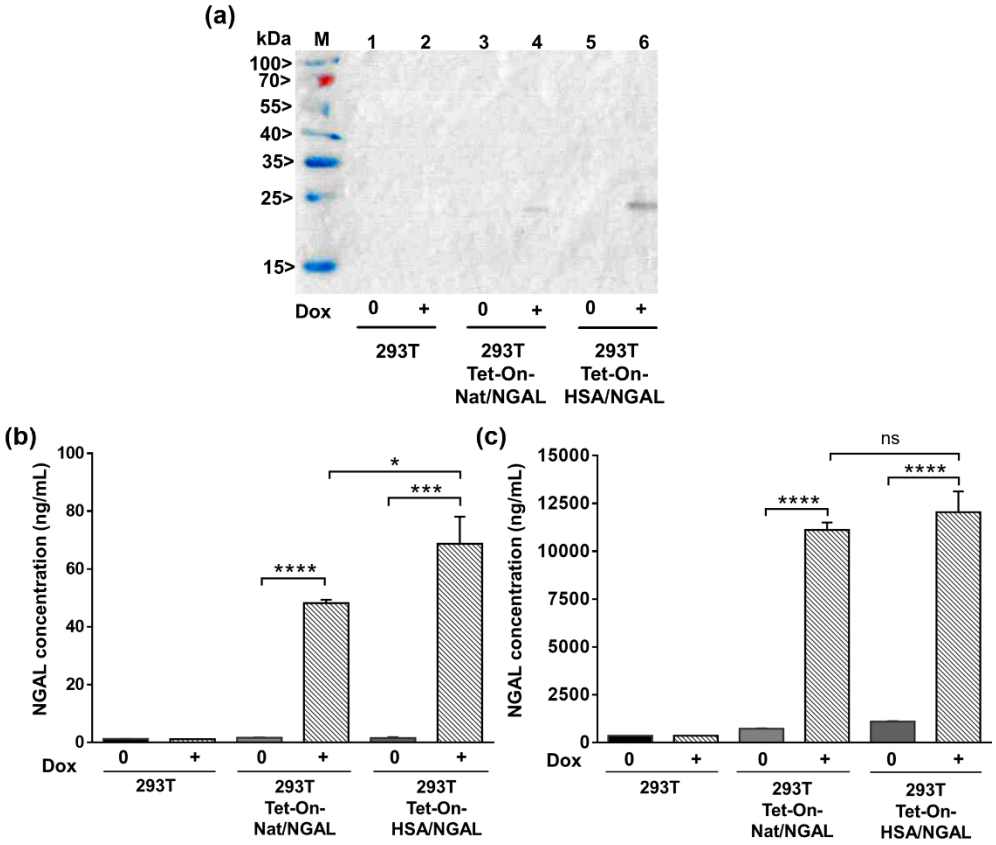

Figure 4. The expression of the NGAL protein in culture supernatants and cellular lysates. (**a**) Detection of NGAL in culture supernatant by Western blot analysis using anti-His tagged monoclonal antibody. Lane 1 and 2 showed 293T control cells with and without Dox. Lane 3 and 4 showed 293T Tet-On-Nat/NGAL with and without Dox. Lane 5 and 6 showed 293T Tet-On-HSA/NGAL with and without Dox, respectively. (**b**) The concentrations of secreted NGAL in the culture supernatant and (**c**) the level of cellular NGAL were measured by ELISA. The results were obtained from triplicate experiments (mean ± SD). Data were analyzed using Student's *t*-test (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$).

4. Discussion

The overexpression of foreign proteins without regulation results in protein accumulation and leads to cell death [27,28]. Several factors have been implicated elsewhere such as the accumulation of toxic metabolites and by-products, inappropriate culture conditions, and unsuitable secretory machinery. To overcome this problem and to enhance the level of secreted protein production, the modulation of recombinant protein expression and the appropriate signal peptide are required.

NGAL has demonstrated its functions including the induction of apoptosis, suppression of bacterial growth, and transportation of small molecules into cells. Focusing on NGAL-induced apoptosis, it is mediated by 13-*cis* retinoic acid treatment within human sebaceous glands [29]. Homologous proteins in the mouse, 24p3, binds to its receptor mediating intracellular iron depletion, leading to cell apoptosis [30]. The viability of NGAL-expressing cell lines during protein production processes is important, and the accumulation of NGAL must be the major consideration.

In the present study, we combined two strategies including the regulation of protein expression by the Tet-On system and the optimization of secretory machinery by inserting an effective signal peptide. The result showed that our production process for NGAL protein was able to be controlled in a Dox-dependent manner without cellular toxicity. This protein was also able to be recognized by antibodies against NGAL used in a commercial ELISA test kit indicating its relative conformational structure to the native NGAL protein. Remarkably, the HSA signal peptide promoted protein secretion related to the previous

study. Of note, this signal peptide not only improves the protein secretion but also remains the biological activity of NGAL protein in terms of the iron-binding molecule [24]. The translocation of secretory proteins into the endoplasmic reticulum depends on a particular limiting step. There are alternative signal sequences that are able to augment the protein secretion [31]. The signal sequences derived from human serum albumin and azurocidin have been demonstrated as efficient machinery for the transient and stable expression of antibodies [32,33].

A low background of EGFP expression was observed in the absence of Dox conditions in this work. The leakage of transgene expression in the non-induced state of Tet promoters has been documented elsewhere [34]. Several strategies to minimize basal leakiness of transgene expression have been developed. The mutations in rtTAs augment the transcriptional activity and Dox sensitivity. These rtTA variants show no activity in the absence of Dox [35]. Recently, the TetRI194T mutation has demonstrated a superior performance [36]. According to the culture process, contamination of the cell culture serum with tetracycline occurs regularly, which is one drawback of the tetracycline-induced operator system. Thus, the use of tetracycline-free fetal bovine serum or serum-free medium can optimize the system.

Internal ribosome entry site (IRES)-based bicistronic vectors have been used in several studies [37–39]. IRES elements allow the expression of two genes in a single transcript. The element contains a single promoter with two cistrons separated by an IRES sequence that permits eukaryotic ribosomes to initiate a 5′-cap-independent translation of the second cistron gene [40]. The major advantages of IRES in this work are: (i) the expression of the EGFP reporter gene is independent to NGAL. Therefore, the process to eliminate the reporter protein is not necessary; (ii) the expression of EGFP indicates that such cells secrete NGAL, which could be appropriate for positive selection of the single NGAL-expressing clone; and (iii) the expression of EGFP indicates not only transduction efficiency but also NGAL expression under the same controllable promoter.

5. Conclusions

In this study, the production of NGAL protein in 293T cells can be optimized under a Tet-On regulation system and HSA signal peptide. Although this work has been set up in a small scale to demonstrate its feasibility, the production level could be scaled up with optimum conditions of Dox induction and tested for its biological activities in the future. Therefore, this approach has paved the way to further generate stable cell lines using lentiviral technology regulated with Tet-On.

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