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Expression of fluorescent tagged recombinant erythroferrone protein

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ABSTRACT

Objective: To produce fluorescent tagged recombinant erythroferrone protein (ERFE_eGFP) for laboratory investigations. **Methods:** Erythroferrone (*ERFE*) gene was fused to green fluorescent protein (*eGFP*) gene and cloned in a pSecTag2Hygro plasmid. The constructed plasmid was amplified in *Escherichia coli* DH5 α and the eGFP-fused ERFE (ERFE_eGFP) protein was expressed in human embryonic kidney (HEK293T) cell line. **Results:** The plasmid constructed from colony C6 contained ERFE_eGFP with the correct restriction sizes of 4.2 kb and expressed secretory ERFE_eGFP fusion protein (approximately size of 75 kDa) in HEK293T cell line. **Conclusions:** ERFE_eGFP recombinant protein is successfully expressed as a secretory functional protein and could be sensitively detected using fluorometry. This fusion protein might benefit future applications for localization of cellular ERFE receptors and competitive immunoassay of ERFE concentration.

1. Introduction

Iron acts as a key player in cell metabolism and proliferation by regulating enzymatic activity and oxidation-reduction reaction. Excessive irons cause oxidative tissue damage by generating toxic reactive oxygen species that activate reticuloendothelial system, leading to aggravating inflammatory process. Iron deficiency may also lead to cell damage and death[1]. Therefore, iron levels need to be tightly regulated by the body to prevent iron excess or iron deficiency. Iron homeostasis depends on three main cellular processes such as iron absorption, iron storage and iron uptake. The peptide hormone hepcidin orchestrates these processes. Hepcidin controls the major flows of iron into plasma at the absorption of dietary iron

in the intestine and at recycling of iron by macrophages. Iron is exported from these tissues into plasma through ferroportin, the iron exporter, which is also known as hepcidin receptor[2–4]. Hepcidin binding to ferroportin causes endocytosis and degradation of iron, leading to the retention of iron in iron-exporting cells and decreased flow of iron into plasma[5].

In turn, hepcidin production is transcriptionally regulated in response to changes in circulating iron concentration, iron stores or the development of inflammation and iron-restricted erythropoiesis[6–8]. Although hepcidin expression is increased in infection through IL-6,

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its level is decreased in severe anemic patients[9–11]. Ineffective erythropoiesis decreases hepcidin expression and causes iron overload in the body[12]. Some studies showed hepcidin gene suppression was caused by bone marrow secreted growth differentiation factors 15[13,14] and twisted gastrulation protein homolog 1[15]. However, they are not significant in other studies[16].

Erythroferrone (ERFE) is a new erythroid regulator peptide hormone which is responsible for hepcidin suppression[17,18]. ERFE is produced from erythropoietic tissues such as bone marrow and spleen by the action of erythropoietin and it acts on the hepatic cell by binding its receptor on the cell surface. However, the ERFE receptor and its downstream signaling molecules have not yet been identified. Therefore, the sufficient production of recombinant ERFE protein will be the foundation of further investigations, especially the molecular mechanisms during activation of ERFE receptor upon ERFE binding. One possible alternative to produce useful ERFE is to conjugate ERFE with fluorescent emitted substances *in vitro* or tagged fluorescent protein *in vivo*. This fused molecule would facilitate visualization of the binding of ERFE to its receptor. Hence, this study aimed to express ERFE in human embryonic kidney 293 (HEK293T) cell line in the form of fusion protein with green fluorescent protein (ERFE_eGFP).

2. Materials and methods

2.1. Construction of plasmid containing *Homo sapiens* ERFE and enhanced ERFE_eGFP genes

Plasmid pcDNA3.1+ that contains human erythroferrone gene (*ERFE*, *FAM132B*) with *FLAG* tag (DYKDDDDK) (pcDNA3.1+ERFE) was purchased from OriGene. Both ends of the 1 065 base pairs *ERFE-FLAG* gene fragment were modified by polymerase chain reaction (PCR) amplification, using pSecERFE_F and ERFE_Linkers_R primers and pcDNA3.1+ERFE plasmid as template, to contain overlapping fragments to pSecTag2Hygro/PSA plasmid and to a cross-linker (GGGSGGGGS).

pSecERFE_F

5' ACTATAGGGAGACCCAAGCTGGCTAGCATGGCCCCGGC
CCGCCG 3'

ERFE_Linkers_R

5' CTACCTCCACCTCCACTACCACCTCCACCCACGCCAGG
AGGACAGC 3'

The 717 base pairs of *eGFP* gene fragment were amplified by PCR reaction using pUb-eGFP plasmid as a template. Both ends of PCR sequences were extended by using a forward primer that contains overlapping region of cross-linker (GGGSGGGGS) and by using a reverse primer that contains overlapping region of pSecTag2Hygro/PSA plasmid.

Linker-eGFP_F

5' AGGTGGTAGTGGAGGTGGAGGTAGTATGGTGAGCAAGG
GCGAG 3'

eGFP-pSec_R

5' GATGAGTTTTTGTTCGGGCCCTCTAGACTTGACAGCTC
GTCCATG 3'

pSecTag2Hygro/PSA (6 408 bp) plasmid was digested with *Nhe* I and *Xba* I to remove IgA kappa chain with *PSA* gene (838 bp). After confirmation on 0.7% agarose gel electrophoresis, *ERFE* gene, *eGFP* gene and *Nhe* I//*Xba* I-digested pSecTag2Hygro plasmid backbone were assembled by Gibson assembly technique (Figure 1)[19]. Briefly, all of the three fragments were equally mixed together with Gibson 2X reagents and incubated at 50 °C for 30 min. The constructed plasmid DNA was transformed into DH5 α *Escherichia coli* which were subsequently plated on LB agar plates supplemented with ampicillin (100 μ g/mL) and incubated at 37 °C overnight.

2.2. Expression of the recombinant ERFE_eGFP fusion protein in HEK293T cell line

One day before transfection, HEK293T cells (5×10^5 cells) were seeded in each well of a 6-well-plate to reach 70%-90% confluent on the day of transfection. The constructed plasmid (2 g) was added with Opti-MEM-reduced serum (100 μ L) and FuGENE HD transfection reagent (Promega, UK) (7.5 μ L). The mixture was incubated for 15 min at room temperature before being transferred in a drop-wise to the confluent cell layers.

The cells were further grown at 37 °C in 5% CO₂ incubator for 48 h and then observed under a fluorescence microscope. After 48 h, the complete Dulbecco's Modified Eagle Medium was replaced with serum free Opti-MEM medium supplemented with vitamin C (0.1 mg per mL of media). The culture media were collected and pooled every 48 h for 3 times. The media were then concentrated with Amicon Ultra Centrifugal Filter Units (Ultra-15, MWCO10000 Da) and total protein concentration was measured by Bradford's assay.

2.3. Confirmation of the recombinant ERFE-eGFP expression by Western Blot analysis

The sample proteins (30 g) were loaded into each well of the 10% SDS-PAGE gel along with molecular weight marker and run at 300 V, 25 mA for 1.5 h. The proteins from the gel were then transferred to nitrocellulose membrane at 30 V, 400 mA for 1.5 h. The complete transfer of proteins on the membrane were checked with Ponceau-S staining. Next, the membrane was blocked with 5% skim milk in phosphate-buffered saline with 0.05% Tween 20 for 1 h at room temperature. The rabbit anti-GFP IgG antibody (1:1 000) was applied to the membrane which was incubated overnight at 4 °C. The membrane was then washed 3 times with 5% skim milk before the secondary anti-rabbit IgG antibody conjugated with horse-radish peroxidase (1:10 000) was added and incubated at room temperature for 1 h. Finally, the unbound secondary antibody was washed with 5% milk for 3 times and the membrane was developed for signal using Pierce™ ECL Western Blotting Substrate following the kit's manufacturer's recommendations.

3. Results

3.1. Construction of plasmid for expression of recombinant ERFE_eGFP fusion protein

The PCR products of *ERFE*, *eGFP*, and the plasmid backbone (*Nhe* I // *Xba* I -digested pSecTag2HygroPSA) were confirmed on agarose gel prior to assembly. The results showed the expected bands of *ERFE*, *eGFP*, and the plasmid backbone at 1 119 bp, 769 bp, and 6 312 bp, respectively (Figure 1). After following Gibson assembly method and transformation of the assembled plasmid into *DH5* α *Escherichia coli*, colonies of the bacteria were randomly selected for plasmid extraction and restriction digestion to confirm the constructed plasmid. It was revealed that plasmid from colonies C6 and C10 contained ERFE_eGFP with the correct restriction sizes of 4.2 kb and 3.1 kb, respectively (Figure 2 and 3). Plasmid from C6 was then confirmed for the introduced ERFE_eGFP by DNA sequencing and chosen for expression of recombinant ERFE_eGFP in the HEK293T cell line.

3.2. Expression of recombinant ERFE_eGFP fusion protein in HEK293T cell line

Using commercially developed transfection reagent (FuGENE), the transfection efficiency could be determined under a fluorescence microscope by the expression of eGFP. As shown in Figure 4, as well as the positive control plasmid pUb-eGFP, the recombinant ERFE_eGFP fusion protein could be expressed in HEK293T cell line using the constructed pSec_ERFE_eGFP plasmid. The Western blot analysis of the culture supernatant confirmed the expression of ERFE_eGFP at the approximate size of 75 kDa (Figure 5). These results indicated the expression of recombinant ERFE_eGFP fusion protein in HEK293T cell line as a secreted protein.

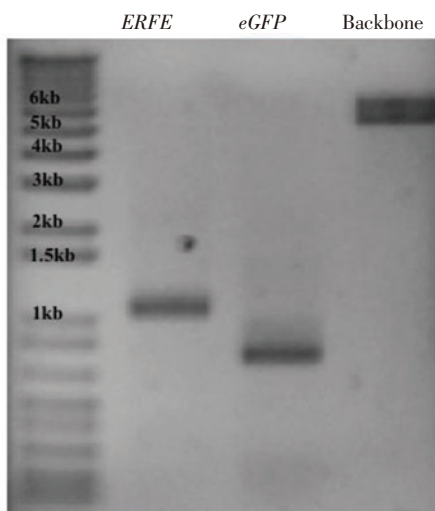


Figure 1. PCR products of *ERFE*, *eGFP* and pSec plasmid backbone (Backbone).

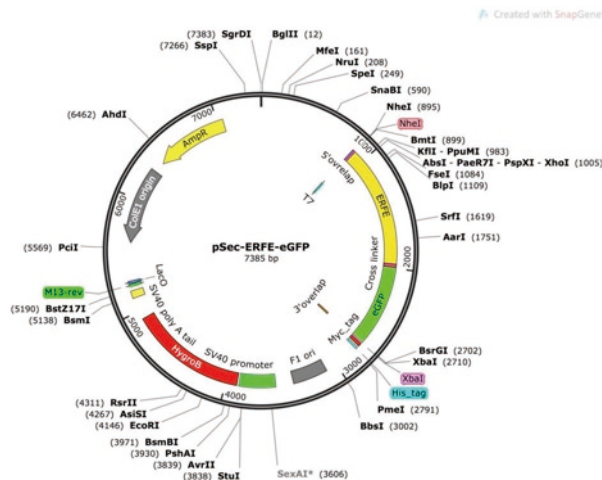


Figure 2. Map of pSecERFE_eGFP plasmid.

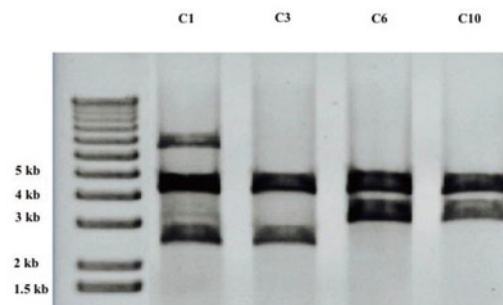


Figure 3. Digestion of plasmid DNA from transformed *DH5* α *Escherichia coli* colonies C1, C3, C6 and C10 with *Xho* I and *EcoR* I restriction enzymes.

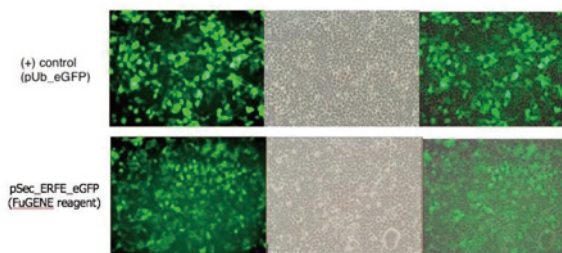


Figure 4. Expression of recombinant ERFE_eGFP fusion protein by HEK293T cell line.

pSec-ERFE_eGFP plasmid was transfected to HEK293T cell using FuGENE transfection reagent. Both of the fluorescent (Fluorescent) and bright (Bright) fields were observed under a fluorescence microscope and the images from both fields were merged (Merge).

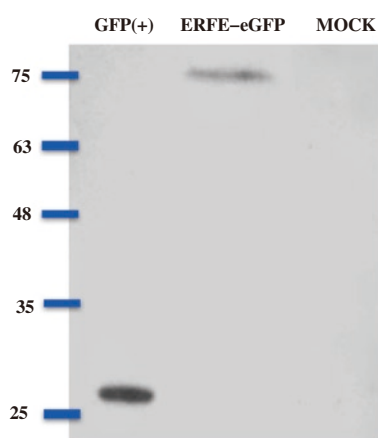


Figure 5. Western blot analysis of recombinant ERFE_eGFP.

Recombinant ERFE_eGFP was expressed in culture media of HEK293T cell transfected with pSec_ERFE-eGFP plasmid, along with standard eGFP protein [GFP(+)] as a positive control and culture media of HEK293T cell with pcDNA3.1+ (MOCK) as a negative control.

4. Discussion

In this study, the plasmid for expression of eGFP-tagged ERFE was successfully constructed using the Gibson assembly technique by which 20-25 base pairs of overlapping region in each target DNA fragment were assembled in one step. The technique has been claimed as a fast and precise technique when compared to the traditional restriction enzyme digestion and ligation[20]. To extracellularly express the recombinant human ERFE-eGFP fusion protein, the signal peptide must be present at the N-terminal of the protein. Although pSecTag2HygroPSA plasmid already has Ig kappa chain signal peptide which is commonly used for extracellular protein expression, we used the native signal peptide present in the ERFE sequence for the natural expression[21].

Although a protein could be expressed in bacteria, yeasts, and other mammalian cell lines such as Chicken Embryonic Ovary, it might not yield the correct form if the protein requires posttranslational modifications. Especially, the glycosylation processes are known to be absolutely important for ligand binding to its cognate receptor. Even though the uses of eukaryotic cells as a host for expression such as yeasts and Chicken Embryonic Ovary, the glycosylation pattern might differ among the host species[22]. In addition, a human cell line like HEK293 can be alternative for the expression to assure the natural posttranslational modifications. However, we chose to express our ERFE-eGFP fusion protein in HEK293T cell line which is most suitable for our expression plasmid that contains SV40 region because it can be replicated in HEK293T cells due to the presence of SV40 large T antigens in the cells[23].

The presence of eGFP tagged to the ERFE was for detection of the ERFE expression, localization, and interaction with target receptor. The GFP protein can be directly tagged into either N or C terminal of the expressed protein, with interruption with uncharged amino

acids to prevent misfolding[24]. In contrast, Medina-Kauwe and colleagues reported expression of the receptor binding domains of Heregulin- α 1 without using short peptides cross-linker between the two proteins[25]. The expressed protein could be further used for monitoring upon binding to the cell surface during endocytosis. Moreover, fluorescent indicator of protein folding showed the tagged proteins fold robustly, soluble and functional[26], and well expressed[27]. On the safe side, our experiment was applied with 10-amino-acid peptide linker (GGGGSGGGGS) between ERFE and eGFP to prevent interfering of folding of each protein. Consequently, the expressed eGFP tagged ERFE proteins emitted fluorescent light brightly under fluorescent microscope to ascertain that the constructed protein was expressed in a correctly folded form.

Apart from eGFP, a fluorophore can be used to conjugate with expressed ERFE to visualize the ligand receptor interaction. Seldin and colleagues reported the expression of ERFE-FLAG (FAM132b) protein in HEK293T for purification by an anti-FLAG affinity column[28]. They further used those expressed ERFE-FLAG in treating the cells to study the role of ERFE in lipid metabolism. In comparison of ERFE-eGFP to ERFE-FLAG protein, the disadvantage of ERFE-eGFP is that eGFP might interfere with the binding of ERFE to its receptor because its size is rather bigger than FLAG peptide (eight amino acids). On the other hand, the advantage of ERFE-eGFP is that it does not need a fluorophore conjugation step, which is essential for ERFE-FLAG, to visualize the ligand receptor binding and to study receptor mediated endocytosis.

Correctly expressed GFP-tagged ligand could be useful for cellular interaction visualization. The visualizing ligand receptor interaction including receptor mediated endocytosis has been reported by the observation under confocal microscopy[29]. It also could be used for sorting cells that contain specific receptor which bind to GFP tagged ligand by using fluorescent activated cell sorting[27]. Our further experiments aim for using GFP, which was tagged in ERFE, in co-immuno-precipitating ERFE-receptor with anti-GFP antibody regardless of ERFE antibody for mass spectrometry.

Although the molecular weight of eGFP tagged ERFE protein according to amino acid compositions was estimated about 65 kDa, the expressed ERFE_eGFP protein in our study was seen at 75 kDa by Western blot analysis. It indicated that our expressed extracellular ERFE_eGFP protein contained three glycosylation sites of ERFE. Therefore, the expressed extracellular ERFE_eGFP containing post translational glycosylations can be potentially used as a functional protein in future applications.

In conclusion, the human ERFE was successfully expressed as eGFP-tagged protein at C-terminal region in HEK293T cells. The protein was exported to the culture supernatant as extracellular protein. The expressed recombinant ERFE-eGFP fusion protein can be applied in future experiments including identification of cellular ERFE receptor, study of ERFE-ERFE receptor mediated endocytosis, and competitive immunoassay of erythroferrone concentration.

Conflict of interest statement

All the authors have no conflict of interests in this study.

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