



Construction and identification of the recombinant hFcεRIα/RBL-2H3 cells

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ABSTRACT

IgE/FcεRI signal pathway plays a crucial role in triggering allergic reactions, and there is no cross-recognition between IgE and FcεRI in human and rats. In order to obtain the hFcεRIα/RBL-2H3 cell line, total RNA was extracted from U937 cells, and the human FcεRIα gene was obtained by RT-PCR technology. Then the amplified product was digested and inserted into the pIRES₂-EGFP vector. After the plasmid was transfected into the RBL-2H3 cells using lipofectamine, and the RBL-2H3 cell lines of stable expression were screened by G418. The transfection efficiency reached 60.45% with optimizing transfection parameters. The last the expression of hFcεRIα was detected by RT-PCR, western blotting and fluorescent microscopy. The present results demonstrated that the pIRES₂-EGFP-hFcεRIα vector was constructed and a stable cell line of hFcεRIα/RBL-2H3 cells was established successfully. This cell line is promising tools for further research on the pathogenesis and drug development of allergic diseases.

1. Introduction

In recent years, allergic diseases have reached epidemic proportions in the world today, affected > 20% of the population and plagued the patients' quality of life in practice (Dominguez-Ortega et al., 2017). The binding of IgE to its high affinity receptor FcεRI plays a key role in the pathogenesis of allergic diseases (Suzuki et al., 2015). The IgE/FcεRI signal pathway has become a focus target for the treatment of allergic diseases (Yalcin, 2014). However, previous studies have suggested that there is species specificity in the IgE and FcεRI interactions between rodents, horses, dogs and rabbits, but not between human and rhesus monkeys. As materials most used in laboratory, rodents and cells from rodents are limited in the researches on allergic diseases due to the species-specific between rodents and humans (Hakimi et al., 1990). Rat basophilic leukemia cell (RBL-2H3) possesses many biological characteristics of mast cells that mimic multiple functions of mast cells, it can be easily cultured and obtained a large number of homogenous cells in vitro (Sun et al., 2015). This cell line has become an important model in vitro for detection of degranulation reaction and screen compounds and researches on mechanism of allergy (Takagi et al., 2003; Rashid et al., 2012).

As a bridge in the interaction of IgE and FcεRI, the hFcεRIα (α-chain of human FcεRI) can be coupled to endogenous β- and γ- chains of

rodents to form a chimeric IgE receptor, which can trigger the activation and degranulation of mast cells (Takagi et al., 2003). In the study, we cloned the hFcεRIα gene and constructed a recombinant eukaryotic expression vector pIRES₂-EGFP-hFcεRIα. The stable cell line hFcεRIα/RBL-2H3 was obtained through optimized conditions. This cell line could be the valuable material for the development of pathogenesis and drugs of allergic diseases.

2. Materials and methods

2.1. Cell culture and reagents

RBL-2H3 cells and U937 cells were purchased from the cell bank the Chinese Academy of Sciences; pIRES₂-EGFP and *DH5α* were preserved in our laboratory; *Bgl* II, *Eco*RI, T4 DNA Ligase, Plasmid extraction kit, RNA extraction kit, DNA recovery kit, and Rabbit anti-human FcεRIα antibody were all purchased from Sangon; Human IgE (hIgE), Myeloma was purchased from Calbio chem. Mouse anti-Human IgE Secondary Antibody, PE was purchased from Thermo Fisher. dNTP, PCR Buffer, Taq DNA polymerase, Reverse transcription kit, FITC-labeled goat anti-rabbit IgG, HRP-labeled goat anti-rabbit IgG were from Cwbiotech; DMEM culture medium, G418, trypsin were from Solarbio; Fetal bovine serum were Hyclone; GeneTran III transfection reagent and geneTran II

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high efficiency transfection reagent were obtained from Biomiga; PCR primers were synthesized by Sangon.

2.2. Vector construction

2.2.1. Gene amplification

Primers were designed by searching for the hFceRI α genomic sequence and the restriction site from (GenBank ID: J03605.1). As follows: Upstream primer 5'-GAAGATCTGAAGAAGATGGCTCTGC-3' (25 bp), the underlined part indicates the *EcoRI* restriction enzyme recognition site; and lower reaches. Downstream primer 5'-ssGGAATTC TCAGTTGTTTTGGGGTTTG-3' (28 bp), the underlined part indicates the *Bgl* II, restriction enzyme recognition site. U937 cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum at 37 °C, 5% CO₂. When the cell confluence reached 90%, the total RNA of the cells was extracted, and hFceRI α gene was cloned by RT-PCR method, with parameters as followed: cDNA 1 μ g; 94 °C, 2 min; 94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s; 25 cycles; 72 °C, 2 min. Product of RT-PCR was evaluated by agarose gel electrophoresis and the target fragment was purified with the relevant kit.

2.2.2. Construction of the pIRES₂-EGFP-hFceRI α expression vector

The recovered target gene hFceRI α and pIRES₂-EGFP were digested with *EcoRI* and *Bgl*II, then T4 DNA ligase at 4 °C overnight. The prepared *Escherichia coli* DH5 α competent cells and the connection product by rotary mixing, subsequently applied to LB solid medium containing ampicillin of 1000 μ g/mL and cultured for 12 h. Positive colonies screened were inoculated on LB solid medium containing ampicillin 1000 μ g/mL, and 37 °C 180 r/min shaking culture 12 h. Plasmid extraction according to the instructions of the plasmid kit. In order to further verify the correctness of constructing the plasmid, the above positive plasmids were identified by sequencing, and the result provides a sequence alignment with GenBank. The recombinant vector of human FceRI α was designated as pIRES₂-EGFP-hFceRI α . It was extracted in large scale and reserved.

2.3. Optimizing transfection conditions of RBL-2H3 cells and screening of the stable cell line

2.3.1. RBL-2H3 cell transfection

RBL-2H3 cells were maintained in DMEM medium containing 10% fetal bovine serum at 37 °C, 5% CO₂. One day before transfection, plated 5×10^5 cells in a 6-well format with growth medium without antibiotics and incubated for 24 h. When the degree of cell confluence reaches 90–95%, the cells are transfected to prepare the transfection complex. 50 μ L the non-serum DMEM medium were diluted with the proper amount and incubated at room temperature for 5 min, then mix the two solutions gently and incubate at room temperature for 20 min. Finally, each drop was added to the orifice plate, then mixed with the orifice plate before and after shaking, incubated in 37 °C, 5% CO₂ for 24 h. Transfection efficiency was detected by counting the RBL-2H3 cells with fluorescent expression by the fluorescence microscope at 24 h after transfection.

2.3.2. Optimization of cell number

One day before transfection, plated 1×10^5 , 5×10^5 and 1×10^6 cells in a 6-well format, respectively, with three duplications each group. The confluence of cells was observed after 24 h. Transfection efficiency was detected by the method mentioned above.

2.3.3. Optimum transfection time of RBL-2H3 cells

The day before transfection, different numbers of cells was seeded in 6-well plates. After 24 h, complexes of DNA and transfection reagent were prepared, then added into cells according to the manufacturer instruction. Incubated for different times (3 h, 4 h, 5 h, 6 h and 7 h), and then changed medium with fresh DMEM containing 10% fetal bovine

serum. Transfection efficiency was detected by the method mentioned above.

2.3.4. Optimization of the ratio of plasmid and transfection reagent

According to the best cell number and transfection time, the different amount of plasmid (3.0 μ g, 4.0 μ g, 5.0 μ g and 6.0 μ g), the proportion of plasmid and transfection reagent (1:1, 1:2, 1:3, 1:4) were prepared according to the manufacturer instruction respectively. Transfection efficiency was detected by the method mentioned above.

2.3.5. Screening of stable recombinant cell line

RBL-2H3 cells were maintained in DMEM medium containing 10% fetal bovine serum in a 24-well format at 37 °C, 5% CO₂. the culture medium was replaced with the fresh medium containing 0 μ g/mL, 300 μ g/mL, 400 μ g/mL, 500 μ g/mL, 600 μ g/mL, 700 μ g/mL, 800 μ g/mL and 900 μ g/mL of G418 with the confluence of 50%, Each concentration was 3 compound pores, and medium was replaced with the medium containing the same concentration of G418 every 2–3 days. The minimum concentration of G418 was selected after incubating after 12 days, which could kill all the cells.

The transfected RBL-2H3 cells were incubated in the medium and the next day the culture medium was replaced with the fresh medium containing the minimum concentration of G418. Every 3 days, the medium containing G418 was replaced, and all the cells in the transfection group died. The cells were cloned by limited dilution and the clones with fluorescent expression by the fluorescence microscope were selected, and the half of the minimum concentration of G418 was used to maintain the screening.

2.4. Identification of transfected RBL-2H3 cells with hFceRI α

2.4.1. Identification of hFceRI α gene expression by RT-PCR

The cells were collected after 24 h transfection, and the total RNA of cells transfected with pIRES₂-EGFP-hFceRI α and transfected pIRES₂-EGFP were extracted by RNA extraction kit, respectively. Total RNA was isolated from transfected cells and target DNA was identified by RT-PCR, with β -actin as internal standard. The primer as follow: top primer 5'-CTCCATCCTGGCCTCGTGT-3' (20 bp), reverse primer 5'-GCTGTACCTTACCGTTCC-3' (20 bp), the PCR product should be 308 bp.

2.4.2. Identification of hFceRI α expression with Western blotting

The membrane protein of transfected RBL-2H3 cells was extracted and identified by Western blotting. Membrane Protein was transferred to acetic acid cellulose membrane through SDS-PAGE electrophoresis, and then blocked overnight at 4 °C. The protein was then incubated with anti-hFceRI α antibody at the rate of 1:500 at 37 °C for 1 h, and washed twice with TBST followed by incubation with HRP-conjugated goat anti-rabbit IgG at the rate of 1:2000 at 37 °C for 1 h. The cells were washed twice and then developing reagent was added. The membrane was rocked gently until the bands could be seen clearly, stopped developing and observed the results.

2.4.3. Identification of hFceRI α expression with immunofluorescence

After 24 h of transfection, recombinant cells and wild-type RBL-2H3 cells were seeded in 24-well plates, 4×10^5 /mL, 37 °C, 5% CO₂ overnight. After washing three times, 4% formaldehyde was fixed at room temperature for 15 min, washed three times with PBS, closed with 5% BSA for 1 h. 1: 500 dilution of rabbit anti-human FceRI α antibody was added and incubated overnight at 37 °C. After washing three times with PBS, 1:2000 dilutions of FITC-labeled goat anti-rabbit IgG was incubated for 2 h. After washing three times with PBS, results were observed with the fluorescence microscope.

2.4.4. Activity detection of hIgE binding to hFceRI α on RBL-2H3

RBL-2H3 cells and hFceRI α /RBL-2H3 cells were grown in 24-well

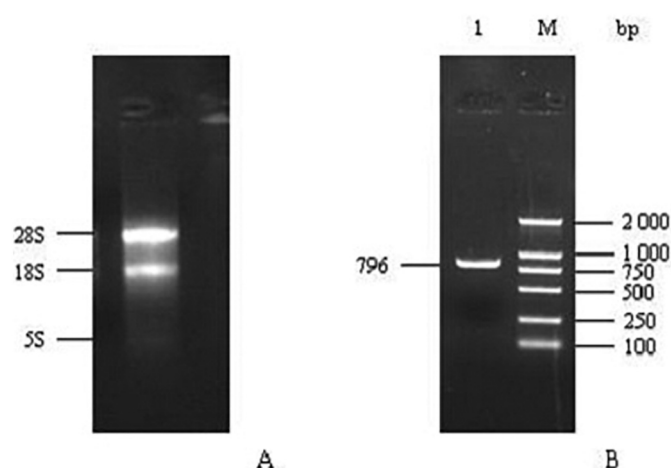


Fig. 1. Analysis of total RNA from U937 and product of RT-PCR. (A) Two bands of 18S and 28S RNA were clearly visible, indicating a higher purity of RNA without obvious degradation and genomic contamination; (B) M: DM 2000 DNA Marker; 1: hFcεRIα gene.

plates (5×10^4 cells/mL), washed twice with PBS, and then incubated with hIgE (0 or 2 $\mu\text{g/mL}$) for 12 h, respectively. The cells were exposed to PE-labeled mouse anti-hIgE antibody (2 $\mu\text{g/mL}$) for 1 h. Then the degranulation was assayed through detecting the release of β -hexosaminidase using an optimized method (Zeng et al., 2016).

2.5. Statistical analysis

All data are presented as mean $\pm$ standard deviation (SD). Multiple student's *t*-test was used to evaluate statistical differences. Indicated significant difference was as $*p < 0.05$, and n.s. stands for no significance. All images shown were representative of the entire result of relevant experiment.

3. Results

3.1. Clone of human FcεRIα

Total RNA was isolated from U937 cells (Fig. 1A). The three bands of 28S, 18S and 5S RNA can be clearly observed. Using the extracted RNA as a template, the target gene was cloned by RT-PCR and the product was 796 bp (Fig. 1B). After comparing with the consequence from GenBank J03605.1, the results showed that the recombinant vector was achieved successfully.

3.2. Construction of pIRES₂-EGFP-hFcεRIα expression vector

The recombinant plasmid pIRES₂-EGFP-hFcεRIα was digested by the *Bgl* I and *Eco* R I. A 796 bp amplified product was observed, which is consistent with the ideal results (Fig. 2). The result showed that recombinant plasmid pIRES₂-EGFP-hFcεRIα was obtained successfully.

3.3. Optimization of transfection conditions of RBL-2H3 cells

Inoculated with 1×10^5 , 5×10^5 and 1×10^6 cells plated in a 6-well format for 24 h, respectively. It was found that the confluence of each group was 60–70%, 75–85% and 90–95%, values are the mean of the duplicate analysis transfected plasmids into RBL-2H3 cells according to protocols previously described. The result showed that the transfection efficiency could be 55.73% with the influence of 75–85% (Fig. 3).

Plated 5×10^5 cells in a 6-well format for 24 h and then prepared complexes with DNA of 4.0 μg and transfection reagent of 10 μL , and

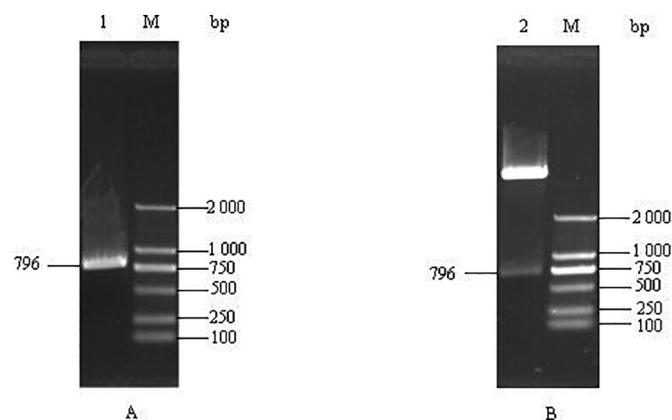


Fig. 2. Identification of the recombinant plasmid by PCR amplification. (A) hFcεRIα gene PCR product. 1: hFcεRIα; M: DM 2000 DNA Marker; (B) Recombinant plasmid pIRES₂-EGFP-hFcεRIα analyzed by restriction digest with *Bgl* II and *Eco* R II. 2: pIRES₂-EGFP-hFcεRIα; M: DM 2000 DNA Marker.

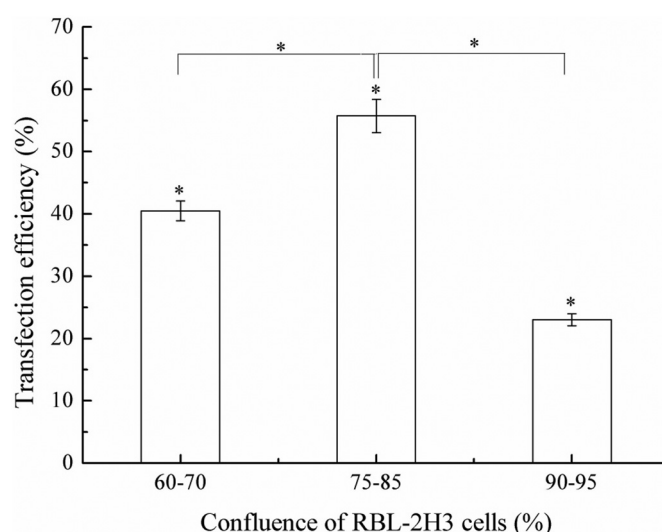


Fig. 3. Effect of cell confluence on the transfection efficiency. RBL-2H3 cell was transfected at different confluence of cells (60–70%, 75–85%, 90–95%). The best high transfection efficiency is 75–85%. Differences were considered significant at the level of $*P < 0.05$.

then mixed together and incubated. RBL-2H3 cell was incubated with transfection reagent at 37 °C for different times (3 h, 4 h, 5 h, 6 h and 7 h) at the same confluence of cells according to the manufacturer instruction. The result showed that transfection efficiency could be 56.41% when incubated for 5 h (Fig. 4).

Plated 5×10^5 cells in a 6-well format and transfected it with different amount of DNA and transfection reagent after 24 h. Complexes were prepared with different amount of DNA (3.0 μg , 4.0 μg , 5.0 μg and 6.0 μg) and liposome (the ratio of DNA and liposome was 1:1, 1:2, 1:3 and 1:4, respectively), and then were used to transfected RBL-2H3 cell according to the manufacturer instruction. The result showed that transfection efficiency could be 60.45% with the ratio of DNA and transfection reagent 1:3 and 4.0 μg DNA (Fig. 5) and that the trend of transfection efficiency was increased at first and decreased afterwards.

3.4. Optimum screening concentration of G418 medium

RBL-2H3 cells was maintained in medium containing 10% fetal bovine serum and various concentration of G418 (0–900 $\mu\text{g/mL}$) at 37 °C and in a 5% CO₂ incubator, and the medium was replaced with fresh medium containing the same concentration of G418 every two

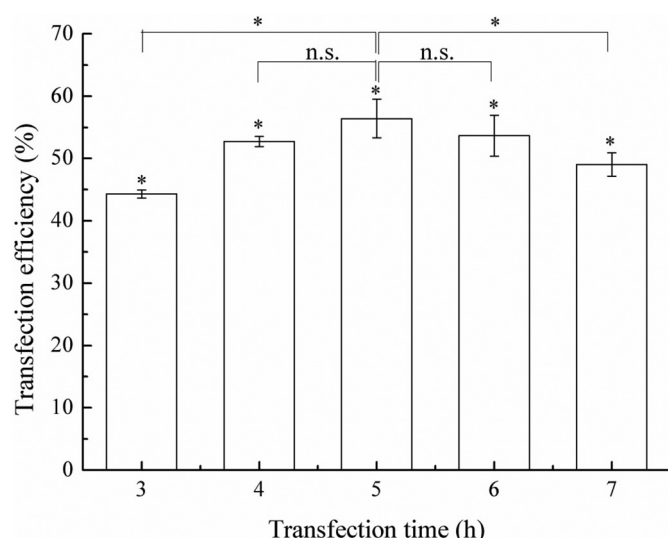


Fig. 4. Effect of transfection time on the transfection efficiency. RBL-2H3 cell was incubated with transfection reagent at 37 °C for different times (3 h, 4 h, 5 h, 6 h, 7 h). The best time is the 5th hour. The results are reported as the means $\pm$ SD.

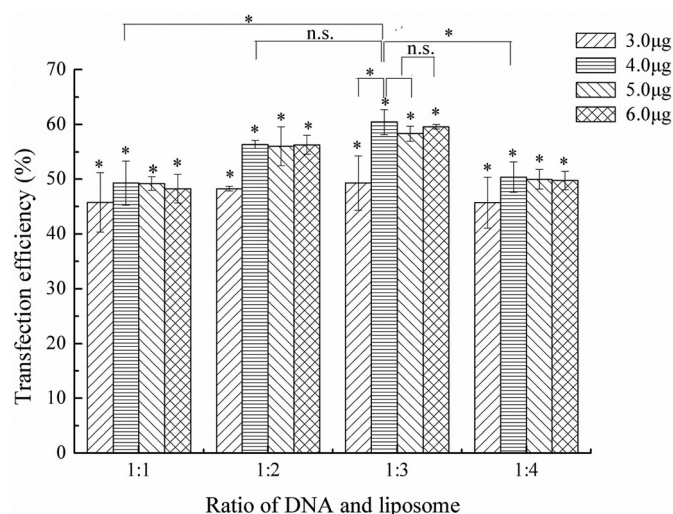


Fig. 5. Effect of DNA and transfection reagent on transfection efficiency. With different amount of DNA (3.0 µg, 4.0 µg, 5.0 µg, 6.0 µg) and liposome (the ratio of DNA and liposome was 1:1, 1:2, 1:3, 1:4, respectively), The best ratio of transfection reagents is 1:3 and the amount of DNA added is 4.0 µL. The results are the average $\pm$ SD of experiments.

days. At first, the cells were in good condition. Treated with 800 µg/mL and 900 µg/mL of G418 for 3 days, cells were beginning to die, with the confluence of control group reaching 90%; 6 days later, the cells treated with 800 µg/mL and 900 µg/mL of G418 almost died and cells with 600 µg/mL and 700 µg/mL of G418 were beginning to die; 9 days later, cells with 700 µg/mL of G418 almost died and cells with 600 µg/mL of G418 were adherent; 12 days later, cells with 600 µg/mL of G418 almost died completely and others died partly. Finally, 600 µg/mL G418 were selected as the optimum concentration (Fig. 6). Passaged transfected cells at a dilution of 1:10 into fresh growth medium when transfected for 24 h and added selective medium with 600 µg/mL of G418 the next day. RBL-2H3 Cells were observed on day 6, day 9 and day 12, respectively.

3.5. Identification of hFcεRIα/RBL-2H3 cell line

After transfection, the expression of hFcεRIα was detected by RT-PCR and Western blotting analysis, both of which showed β-actin expressed in the three kinds of cells (hFcεRIα/RBL-2H3, pIRES2-EGFP/RBL-2H3, wild RBL-2H3). But the hFcεRIα gene could be expressed only in the hFcεRIα/RBL-2H3 cell (Fig. 7 and Fig. 8, respectively).

After transfection for 24 h, the RBL-2H3 cells transfected with pIRES2-EGFP-hFcεRIα showed green positive signals, hFcεRIα gene could be expressed in the hFcεRIα/RBL-2H3 cells successfully (Fig. 9A).

3.6. Activity detection of hIgE binding to hFcεRIα on RBL-2H3

β-Hexosaminidase release has frequently been used as a marker for monitoring degranulation of RBL-2H3 cells. RBL-2H3 and hFcεRIα / RBL-2H3 cells were incubated with hIgE/ anti-hIgE, respectively. The β-hexosaminidase releasing in hFcεRIα / RBL-2H3 cells was significantly higher than other three groups. (Fig. 10) Since there was no hFcεRIα subunit on the surface of RBL-2H3, hIgE could not bind to rFcεRIα subunit and produce the β-hexosaminidase.

4. Discussion

The binding of IgE to the high-affinity IgE receptor (FcεRI) on the surface of target cells can trigger a series of signal transductions in the cells and then mast cells degranulation, which are the critical pathogenicity of allergic diseases (Liu et al., 2008). RBL-2H3 (Rat basophil leukemia, RBL), a subfamily of rat basophil tumor cell line, has been widely used as an early stable and sensitive cell degranulation model for allergy, due to the abundant IgE high-affinity receptors on its membrane and similar characters with mast cells. However, Takagi et al. (2003) have proved that the interaction IgE and FcεRI has species specificity between human and rodents. Therefore, it is important and meaningful to construct a stable hFcεRIα/RBL-2H3 cell line. The present study constructed the eukaryotic recombinant pIRES2-EGFP-hFcεRIα plasmid and determined the effects of hFcεRIα expression of RBL-2H3 cells, and establishment of hFcεRIα/ RBL-2H3 cells model.

As a bridge in the interaction of IgE and FcεRI, the extracellular α subunit is sufficient for IgE binding to FcεRI in the absence of β and/or γ subunit, indicating that the α subunit alone can play a key role in the biological functions of FcεRI with high affinity and specificity to IgE. In this study, the α subunit of human FcεRI was transfected into RBL-2H3, the transfection efficiency of pIRES2-EGFP-hFcεRIα can be observed by the fluorescence microscopy. Through G418 antibiotic selection, a highly and stable expressed hFcεRI cell line was obtained in this paper (Zhu et al., 2018). Recently, some researches showed that different cell transfection parameters and transfection efficiency are different (Elena et al., 2009). Ghomari et al. (1994) also had optimized the conditions of transfection with liposomes and found that the optimal transfection efficiency was achieved by changing the proportion of liposome transfection reagents and the sum cells. In this study, we attempted to transfect RBL-2H3 cells using lipofectamine and by optimizing the number of cells inoculated and the proportion of plasmids to improve transfection efficiency proper confluence of RBL-2H3 cells. However, we were found that with the increase in DNA content and the increase in the ratio of DNA to liposome, the transfection efficiency firstly increased and then decreased, this results were similar with previous reports (Karmali et al., 2006). We thought the reasons probably that DNA and lipofection were relatively insufficient firstly and after saturation of DNA, the toxic effects of liposomes on cells play an important role in the transfection efficiency.

In this study, we successfully constructed the vector pIRES2-EGFP-hFcεRIα plasmid which is different from other eukaryotic expression vectors. The vector contains a reporter gene, the enhanced green fluorescent protein, which emits green fluorescence (Glinka et al., 2009). Because of its Ser65 Thr instead of Phe64 replaced by Leu, EGFP

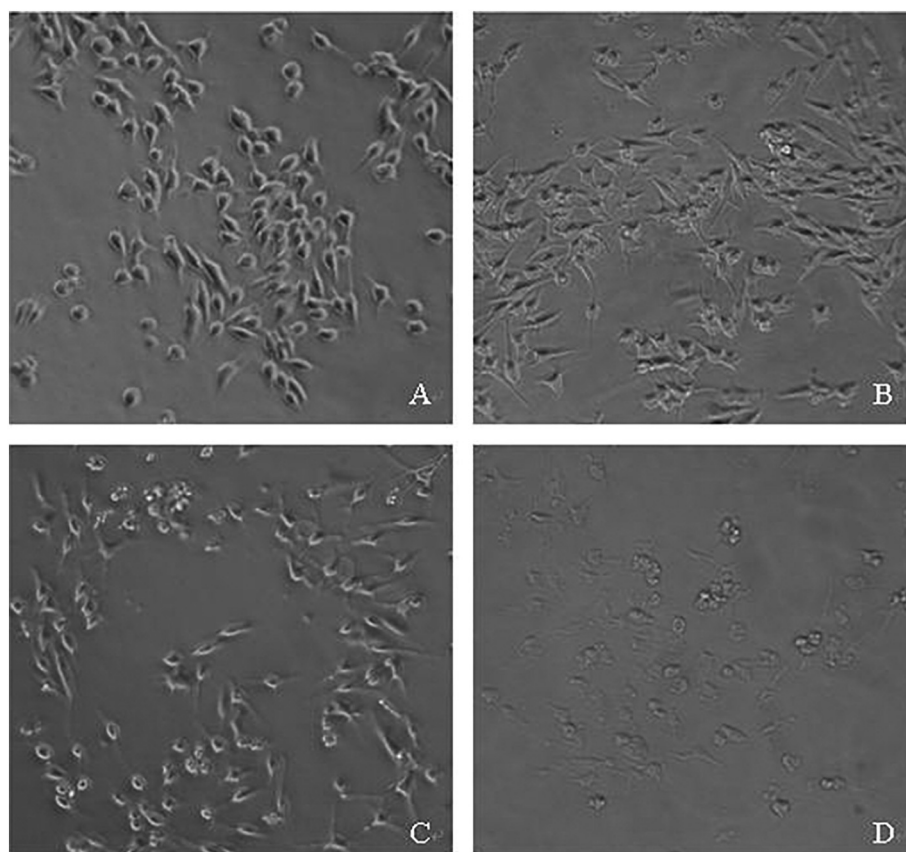


Fig. 6. Conditions of RBL-2H3 cells treated with 600 $\mu\text{g}/\text{mL}$ G418 at various time points. (A) Cells treated with no G418; (B) some cells died after 6 days; (C) some cells died and poor cell status after 9 days; (D) the cells all died after 12 days.

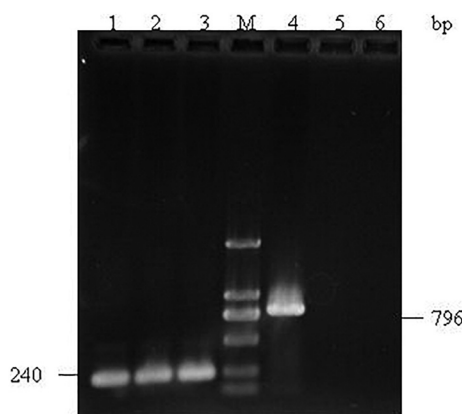


Fig. 7. Expression of hFcεRIα in hFcεRIα/RBL-2H3 by RT-PCR analysis. 1–3: β -actin expressed in RBL-2H3 transfected with pIRES₂-EGFP-hFcεRIα, RBL-2H3 transfected with pIRES₂-EGFP and RBL-2H3, M: 2000 DNA Marker; 4–6: hFcεRIα expressed in RBL-2H3 transfected with pIRES₂-EGFP-hFcεRIα, RBL-2H3 transfected with pIRES₂-EGFP and native cells.

fluorescence intensity increased 35-fold, with ordinary fluorescence microscope to observe its expression, and transfection of 16–24 h after the stable expression (Xu et al., 2017; Wang et al., 2000). The vector contains SV40 ori copies of the elements, the host cell division, to ensure the stability of the target gene transfer; containing IRES, can enhance the level of target gene expression (Tan and Hu, 2013; Zhang et al., 2005). Since the eukaryotic expression vector used contains EGFP, its expression can be directly observed with a fluorescence microscope. The results of this experiment showed that under the fluorescence microscope, the RBL-2H3 cells stably transfect with the green

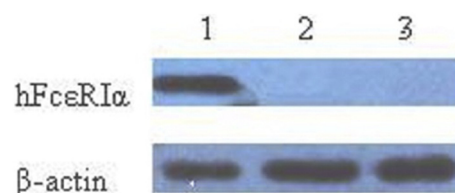


Fig. 8. Expression identification of hFcεRIα in hFcεRIα/RBL-2H3 by Western blotting. Lane 1: Transfected with pIRES₂-EGFP-hFcεRIα; lane 2: Transfected with pIRES₂-EGFP; lane 3: wild RBL-2H3 cells. The β -actin was used as internal standard.

fluorescent protein showed a clear green fluorescence, indicating that the recombinant vector has been successfully transferred into RBL-2H3 cells. The human-rat chimeric IgE receptors can be expressed on RBL-2H3 cells transfected with hFcεRIα, hIgE can bind to the chimeric FcεRI and have the biological activity.

In conclusion, we have constructed the recombinant eukaryotic expression plasmid pIRES₂-EGFP-hFcεRIα, containing human hFcεRIα gene. The stable cell line of hFcεRIα/RBL-2H3 was obtained under the optimized transfection parameters and hFcεRIα gene could be over-expressed successfully. This study provides an experimental tool for the further study on allergy diseases.

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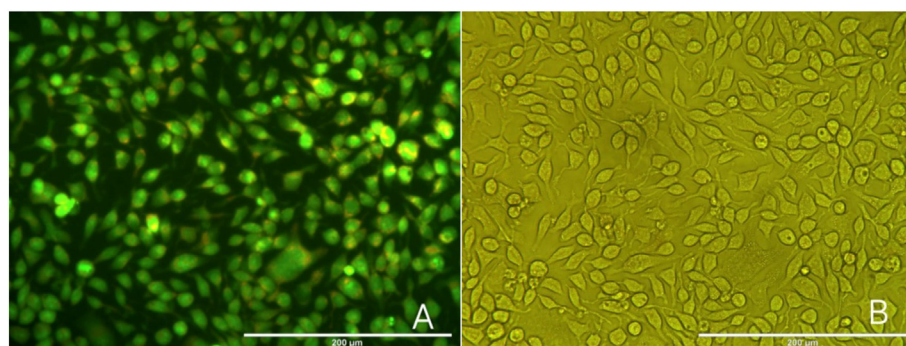


Fig. 9. Detection of the hFcεRIα/RBL-2H3 cells by fluorescence microscopy ($\times 200$). (A) hFcεRIα/RBL-2H3 cells exhibited green fluorescence; (B) phase contrast. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

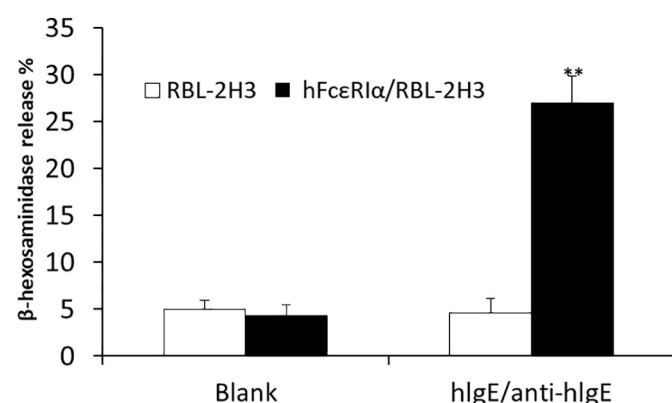


Fig. 10. Detection of hlgE binding to hFcεRIα on RBL-2H3.

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