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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 pIRES$_2$-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 pIRES$_2$-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; pIRES$_2$-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 Therom 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 synthetized by Sangon.

2.2. Vector construction

2.2.1. Gene amplification

Primers were designed by searching for the hFcεRIα genomic sequence and the restriction site from (GenBank ID: J03605.1). As follows: Upstream primer 5'-GAAGATCTGAAGAAGATGGCTCCTGC-3′ (25 bp), the underlined part indicates the *Eco*RI restriction enzyme recognition site; and lower reaches. Downstream primer 5'-ssGGAATTC TCAGTTGTTTTTGGGGTTTG-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% CO_2. When the cell confluence reached 90%, the total RNA of the cells was extracted, and hFcεRIα gene was cloned by RT-PCR method, with parameters as followed: cDNA 1 μg; 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 pIRES$_2$-EGFP-hFcεRIα expression vector

The recovered target gene hFcεRIα and pIRES$_2$-EGFP were digested with *Eco*RI and *Bgl*II, then T4 DNA ligase at 4 °C overnight. The prepared *Escherichia coli* DH5α competent cells and the connection product by rotary mixing, subsequently applied to LB solid medium containing ampicillin of 1000 μg/mL and cultured for 12 h. Positive colonies screened were inoculated on LB solid medium containing ampicillin 1000 μg/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 FcεRIα was designated as pIRES$_2$-EGFP-hFcεRIα. It was extracted in large scale and reserved.

2.3. Optimizing transfection conditions of RBL-2H3cells 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% CO_2. 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 μL 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% CO_2 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 μg, 4.0 μg, 5.0 μg and 6.0 μg), 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% CO_2. the culture medium was replaced with the fresh medium containing 0 μg/mL, 300 μg/mL, 400 μg/mL, 500 μg/mL, 600 μg/mL, 700 μg/mL, 800 μg/mL and 900 μg/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 hFcεRIα

2.4.1. Identification of hFcεRIα gene expression by RT-PCR

The cells were collected after 24 h transfection, and the total RNA of cells transfected with pIRES$_2$-EGFP-hFcεRIα and transfected pIRES$_2$-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'-CTCCATCCTGGCCTCGCTGT-3′(20 bp), reverse primer 5'-GCTGTCACCTTCACCGTTCC-3′ (20 bp), the PCR product should be 308 bp.

2.4.2. Identification of hFcεRIα 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-hFcεRIα 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 hFcεRIα 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% CO_2 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 FcεRIα 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 hFcεRIα on RBL-2H3

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