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Short communication

Optimisation of electroporation and lipofection protocols to derive the black tiger shrimp cell line (*Penaeus monodon*)

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ABSTRACT

To achieve in creating permanent shrimp cell lines, cellular arrest of primary cells in the culture is needed to be firstly solved. Considering the insertion of some markers affecting cellular proliferation into primary haemocytes in order to produce the black tiger shrimp cell line and the very low percent of transduced cells previously reported in penaeid shrimps, these paved us the way to set up suitable gene delivery protocols to increase percent of transduced cells in the shrimp as our primary aim. In this study, electroporation and lipofection were used to transfer construct plasmids (pLL3.7 plasmids containing CMV promoters and pGL-IE1-126(A)-EGFP plasmids carrying WSSV IE1 promoters) into primary haemocytes. As it was difficult to distinguish between cells expressing EGFP signal and auto-fluorescence of many dead cells occurred by electroporation during the first 72 h of experiment; so, only lipofection was managed to deliver plasmids into primary cells. Surprisingly, numbers of suspected proliferative cells were derived after electroporation with no insertion of immortalising markers. These cells survived *in vitro* for up to 45 days with high rate of cell viability, but the number of viable cells decreased throughout the experiment. In addition, these cells expressed genes and proteins closely related to hyaline cells determined using RT-PCR and western blot. For the lipofection experiment, no green fluorescence signal was detected in any primary cell introduced with these plasmids, suggesting that plasmids were not successfully inserted into cells. Also, a number of primary haemocytes had the apoptotic cell death characteristic within 5 days after lipofection. These possibly result from using inappropriate lipofection protocol and chemical substances. In summary, finding out suitable protocols to elevate the percent of transduced cells is still necessary. Additionally, continuous shrimp cell lines would be possibly established by transforming suspected proliferative cells derived from electroporation in this study.

1. Introduction

To date, the shrimp aquaculture industry has been losing lots of money due to massive shrimp mortalities by some disease outbreaks in the shrimp [1]. To find out the cause of disease and its mechanism of infection that could facilitate in diagnosing the disease and promotion of research progression in the shrimp; therefore, *in vitro* cell culture, which is considered as one of important tools widely used to study in the topics of biology, biotechnology, immunology and toxicology in vertebrate species [2], is needed to be developed. In crustacean, no permanent cell line has been reported. This failure is probably due to the cellular arrest, microbial contamination, lack of suitable culture conditions and lack of the evidence to ensure the successful production of permanent cell lines [3,4]. To succeed in deriving continuous cell lines in the black tiger shrimp, our group believes that cellular arrest must be firstly concerned. Therefore, transformation of the black tiger shrimp cells by introduction of some immortalising markers by using different gene delivery systems in order to generate the stable cell line is of our focus.

In animals, it is well-known that immortalised cell lines often originate from somatic tissues that have some problems in the regulation of normal cell-cycle checkpoint pathways e.g. cellular tumour antigen

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p53 (p53) and retinoblastoma protein (pRb) [5] due to inactivation of these cell cycle controllers by the up-regulation of some oncogenes e.g. avian myelocytomatosis viral oncogene homolog (v-MYC) or oncoproteins e.g. simian virus 40 large T antigen (SV40LT) and human papillomavirus and Epstein-Barr virus (HPV) [5–8], together with the reactivation or up-regulation of telomerase enzyme [7,9], leading to a higher rate of cellular proliferation and the establishment of permanent cell lines [6–8,10]. While, methods basically used to deliver markers of interest into eukaryotic cells for generating the stable cell lines are non-virus (e.g. calcium phosphate precipitation, electroporation, particle gun bombardment, direct microinjection, lipofection) and virus-based vector systems [11]. In the penaeid shrimp, no transformed or immortalised cell line has been established even there are many reports trying to reproduce cell lines by using lipofection, electroporation and viral vectors [12–15]. Only the morphology of cells and the expression of designed genes inserted in transduced cells were determined. To generate transformed cell lines in the penaeid shrimp by a non-virus based vector system, lipofection and electroporation techniques were reported to insert SV40LT and adenovirus type 12 early region 1 A (12 S E1A) into primary lymphoid cells of the black tiger shrimp [15]. In case of the viral-based vector approach, retrovirus was previously managed to insert SV40LT gene into primary lymphoid and ovarian cells of *P. Chinensis* [12,13]. Recently, recombinant baculoviral vectors containing shrimp viral promoters e.g. white spot syndrome virus (WSSV) and infectious hypodermal and haematopoietic necrosis virus (IHHNV) were used to produce some transduced primary cells isolated from the black tiger shrimp both *in vitro* and *in vivo* with the provision of the low rate of transduction efficiency (10–20%) [14]. Although viral mediated transduction is claimed to be the most efficient tool to modify the genetics of the majority of animal cells than other gene delivery methods [14], but there is an evidence showing that transduction efficiency of primary shrimp cells transfected by recombinant baculoviral vectors carrying shrimp viral promoters was still very low (5%) [15]. This possibly means that viral vectors used are not suitable to transduce primary shrimp cells. Taken together, methods and protocols used to deliver required markers into primary cells of the shrimp are still not effective to create stable shrimp cell lines and the results described above lead us to the point that optimisation of gene delivery system to promote the percent of cell transduction in the shrimp must be urgently needed. Together with avoiding the viral ability to transmit their genome into primary shrimp cells; therefore, our primary goal of this study was to figure out suitable transformation protocols for the introduction of designed plasmids into primary haemocytes through a non-virus gene delivery system e.g. liposome-based transfection and electroporation in order to increase the percent of transduced cells in the shrimp.

2. Materials and methods

2.1. Animals

Both male and female *P. monodon* at the age of 4–8 months obtained from Shrimp Genetic Improvement Center, Thailand, were kept at the Center of Excellence for Marine Biotechnology, Chulalongkorn University, Thailand, before use. Animal experiments were managed by following the protocol approved by Chulalongkorn University and BIOTEC Animal Care and Use Committee.

2.2. Haemocyte collection

In brief, the whole surface of shrimp was firstly cleaned using disinfectant and sterile dH_2O before being sprayed with 70% alcohol. Haemolymph was withdrawn into a syringe half filled with 10% sodium acetate and then being centrifuged at 3500 rpm for 10 min at 4 °C. The cell pellet was roughly washed before the supernatant was discarded. Then, cell pellet was resuspended and cultured in the medium (2× Libovitz L-15, 5% (v/v) lactalbumin solution, 100 units/ml penicillin, 100 μg/ml streptomycin, 1%fungisone (v/v) and 10% each of FCS and shrimp meat extract) for 24–48 h at 22 °C. After that, floating cells of primary haemocytes were collected and centrifuged at 1500 rpm for 10 min. Then, cell pellet was further used in Experiment I and II.

2.3. Preparation of plasmids

Backbone plasmid pGL-IE1-126(A) modified from previous paper [16] and EGFP containing plasmid pLL3.7 (Addgene 11795) obtained from Addgene were separately transformed into TOP10F competent cells by heat shock at 42 °C for 45 s. The transformed cells were cultured on LB agar plate with 100 μg/ml ampicillin overnight. Single colony was picked up and cultured in 3 ml LB medium with 100 μg/ml ampicillin for approximately 14 h. Plasmid DNA was extracted using Qiaprep spin miniprep kit (Qiagen) according to the manufacturer's protocol. Plasmid DNA concentration was measured using a nanodrop spectrophotometry (ThermoScientific).

2.3.1. pGL-IE1-126(A)-EGFP preparation

EGFP fragment was amplified from pLL3.7 using a pair of primers; forward primer (NK0001): 5′-AGATCTTGAACCGTCAGATCCGCTAG-3′, reverse primer (NK0002): 5′-TCTAGAAAGTTATTAGGTCCCTCGACG-3’. This pair of primers gives a product of 796 bp with *Bgl* II and *Xba* I restriction sites. After PCR reaction, the sample was subjected to electrophoresis to confirm a specific product. Agarose gel at the location of approximately 796 bp was cut and PCR product was extracted using Qiaquick gel extraction (Qiagen), according to the manufacturer's protocol. Extracted PCR product was subjected to 3′A overhang and ligated to pTz57R/T using Ins TA clone PCR cloning kit (ThermoScientific) at 16 °C overnight. The pTz57 R/T-EGFP plasmid was then transformed into DH5α competent cells and spread on LB agar plate with 100 μg/ml ampicillin overnight. The plate was incubated at 37 °C for 16–18 h before picking up individual colonies. Plasmid DNA was extracted from each colony and subjected to confirm EGFP fragment insertion using PCR and primers as described above. After confirmation of EGFP insertion, the pTz57 R/T-EGFP plasmid was cut by specific restriction enzymes *Bgl* II and *Xba* I. Then, EGFP fragment was purified and ligated into pGL-IE1-126(A) precut with *Bgl* II and *Xba* I. Finally, pGL-IE1-126(A)-EGFP plasmids were confirmed by DNA sequencing (Fig. 1).

2.3.2. Transfection into HEK293T

pGL-IE1-126(A)-EGFP and pLL3.7 plasmids were introduced into human embryonic kidney 293 cells that contains SV40LT (HEK293T) cells using lipofectamine 3000 kit (ThermoScientific) according to the manufacturer's protocol. Transfected HEK293T cells were allowed to grow for 48 h before GFP observation was done under an epi-fluorescence microscope.

2.4. Experimental designs

2.4.1. Experiment I: derivation of primary haemocytes by electroporation

In this experiment, floating cells of primary haemocytes were assigned into two groups: control and electroporation. For the control group, the total of cells around 5×10^6 cells was resuspended in culture medium and incubated in the culture system written above without applying any electrical stimulation. While, the same amount of cells in the electroporation group was done as follows. Firstly, the Nucleofector® solution and supplement were mixed as recommended in the protocol (Lonza). The mixed solution was added into cell pellet and mixed gently. Then, transfer the suspension into cuvette. Insert the cuvette into the Nucleofector® Cuvette holder and apply the electrical stimulation as programmed. Take the cuvette out of the holder once the programme was finished. Immediately, transfer the suspension into culture medium and mix gently. Finally, put the culture vessel into the