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Research Paper

A stable transfected cell line expressing sterol carrier protein 2 (SlSCP2) from the cutworm, *Spodoptera litura*

Li-Li Zhang[1], Tian-Yao Bai[2], Xing Wan[1], Hui-Min Deng[1], Li-Ning Liang[1], Si-Chun Zheng[1], Qi-Li Feng[1] and Lin Liu[1]*

[1]Guangzhou Key Laboratory of Insect Development Regulation and Application Research, Institute of Insect Science and Technology, School of Life Sciences, South China Normal University, Guangzhou, 510631, China.
[2]Institute for Conservation and Utilization of Agro-bioresource in Dabie Mountains, School of Life Sciences, Xinyang Normal University, Xinyang, 464000, China.

*Corresponding author. E-mail: liul@scnu.edu.cn. Tel: +86 20 8521 0024.

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ABSTRACT

Cholesterol is an important cellular membrane component and a precursor of biosynthesis of ecdysteroids in insects. The lack of cholesterol would retard insect development. However, insects cannot synthesize cholesterol *de novo* due to lack of two key enzymes in the cholesterol biosynthesis pathway, and as a result there is need for sterols uptake from food by the insects. Sterol carrier protein (SCP) is a cholesterol-binding protein responsible for cholesterol uptake and transport in insect cells and potential drug target for insecticide development. To study the function of SlSCP2 and to screen efficient and specific insect growth chemical inhibitors that target at this protein, a stably transfected CHO cell line that expresses *Spodoptera litura* sterol carrier protein 2 (SlSCP2) was established. Sequencing and restriction enzyme digestion analysis showed that *SlSCP2* cDNAs was cloned into pcDNA5/FRT vector, which was used to transfect CHO cells, followed by drug resistance selection. Genomic PCR, RT-PCR and Western blot analysis indicated that the *SlSCP2* gene integrated into the genome of the CHO cells and the mRNA and protein of *SlSCP2* were correctly expressed in the transfected cells. The positive cell colonies could stably express the protein after twenty-seven (27) generations. The established transfected CHO cell line with stable expression of SlSCP2 provide a useful cell model for high throughput screening for insect growth inhibitors that target SlSCP2, as well as, for functional study of SlSCP2.

Key words: Sterol carrier protein, cholesterol, stable transfected cell line, insect growth inhibitors, high throughput screening.

INTRODUCTION

The polyphagous lepidopteran cutworm *Spodoptera litura* is a destructive agricultural insect pest in the tropical and sub-tropical areas, feeding on more than two hundred and ninety (290) species of host plants of ninety (90) families, particularly cruciferous vegetables (Zhang et al., 2010). *S. litura* is one member of the holometabolous class of insects. Molting and metamorphosis are mainly coordinately controlled by ecdysteroids, especially active 20-hydroxyecdysone (20E) and juvenile hormone (JH) (Riddiford et al., 1994). 20E initiates the ecdysis process during the larva-to-larva molting and the larva-to-pupa metamorphosis.

Cholesterol is one of the cellular membrane components (Lasser and Clayton, 1966) and a precursor of biosynthesis of ecdysteroids in insects (Ritter and Nes, 1981; Borovsky et al., 1986). The lack of cholesterol would retard the development of insects (Guo et al., 2009). However, insects cannot synthesize *de novo* cholesterol and they have to uptake sterols from food they fed on (Gilbert et al., 2002; Zdobnov et al., 2002; Jouni et al., 2002).

Sterol carrier protein (SCP) is one of the proteins that are responsible for cholesterol uptake and transport in insect cells (Gallegos et al., 2001). SCP2 is an important member of the sterol carrier protein family which shares a common SCP2 domain responsible for the uptake of lipids *in vivo* (Gallegos et al., 2001). Insect SCP2 protein is

encodes by SCP2 or SCPx gene which have the same domain architecture as that of vertebrate SCPx, containing both a thiolase domain and an SCP2 domain (Krebs and Lan, 2003; Lan and Wessely, 2004; Takeuchi et al., 2004; Gong et al., 2006; Ko and Puglielli, 2007; Guo et al., 2009). SlSCP2 can transport a broad range of steroids and lipids, including cholesterol (Ferdinandusse and Houten, 2006; Atshaves et al., 2007; Vyazunova and Lan, 2008; Zhang et al., 2014). Suppressing the expression and function of this protein can result in inhibition of *S. litura* growth and development (Guo et al., 2009), therefore any small chemical compounds that inhibit the function of SlSCP2 and compete with cholesterol for binding with SlSCP2 can be considered as an inhibitor or pesticide. To screen for chemical inhibitors of SlSCP2 using high-throughput technology, a stably transfected cell line that expresses SlSCP2 is a pre-requisite. In this study, a stably transfected CHO cell line that expressed SlSCP2 was developed.

MATERIALS AND METHODS

CHO cell culture and determination of the optimal screening concentration of hygromycin

Chinese hamster ovary (CHO) cell line was provided by the Chinese University of Hong Kong, China. CHO cells were grown in Ham's F-10 medium (Invirogen, Shanghai, China) containing 10% fetal bovine serum (FBS) at 37°C in a CO_2 incubator. The cells were treated with 1 ml of 0.25% trypsin-Ethylene Diamine Tetraacetic Acid (trypsin-EDTA) at intervals of three generations. The cells in the logarithmic growth phase were transferred to the 96-well cell culture plates and diluted with hygromycin-free medium to a cell density of 1×10^4 cells /ml in each well. After 24 h culture, the medium was replaced with fresh Ham's F-10 medium containing 0, 50, 100, 250, 500, 750 and 1000 μg/ml hygromycin, respectively. Cell growth was observed at 12 h intervals until 120 h post primary culture. The cells were then collected and the mortality rate statistically analyzed. At each of the given concentrations, at least three replicates were performed.

Construction of transfection vectors

A *SlSCP2* cDNA was cloned into pMD18-T vector (TAKARA, Dalian, China) and confirmed by sequencing. The open reading frame of the *SlSCP2* was subcloned into the pcDNA5/FRT expression vector (provided by the Chinese University of Hong Kong, China) between the *Kpn* I and *Not* I sites. The *SlSCP2* insertion into the recombinant vector pcDNA5/FRT-SlSCP2 was confirmed using PCR and restriction endonuclease digestion; the vector was therefater used to transfect CHO cells. *GFP* gene was cloned into the pcDNA5/FRT expression vector as a control using the same method as the *SlSCP2*.

Transfection of CHO cell lines with pcDNA5/FRT-SlSCP2 and pcDNA5/FRT-GFP

Cells to be co-transfected were seeded at 2×10^5 cells/ml in 6-well plates and cultured for 12 h. A mixture of 100 μl containing 2 μg pcDNA5/FRT-SlSCP2 or pcDNA5/FRT-GFP expression vector and pOG44 vector at a ratio of 9:1, 15 μl of Lipofectin reagents (Invitrogen) in the serum-free Ham's F-10 medium. After cultured for 8 h, the DNA-containing medium was replaced with 2 ml of fresh Ham's F-10 medium containing 10% FBS and incubated at 37°C. After 24 h in culture, the transfected cells were observed for green fluorescence signals and photographed using a laser confocal microscope.

Western blotting and genomic DNA PCR analysis

For Western blotting analysis, CHO cells cultured for 24 h were collected and cracked in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.0 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% Sodium-dexycholate, protease inhibitor cocktail) for 30 min and thereafter collected and centrifuged at 14,000 × g at 4°C for 25 min. 10 μg of supernatants protein were separated on SDS-PAGE and then transferred to a nitrocellulose membrane. The blots were blocked with 3% BSA in 1× PBS buffer for 2 h at room temperature, and then incubated with the anti-SlSCP2 antibody (1: 1,000) at room temperature for 1 h. Goat anti-rabbit IgG (Dingguo Biotechnology, Beijing, China) conjugated with alkaline phosphatase was used as the secondary antibody at a dilution of 1:1,500. Nitroblue tetrazolium and 5-bromo-4-chloro -3-indolyl phosphate were used as substrates for color development.

Genomic DNA of the cells was isolated and *SlSCP2* was PCR-amplified using the following conditions: 95°C for 2 min, 30 cycles at 95°C for 30 s, 58°C for 30 s, 72°C for 1 min 20 s and final extension at 72°C for 10 min. The *SlSCP2*-specific primers were 5'-GGTACCATGGCCATGTACCGCAAAGGATTCGC-3' (forward) and 5'-GCGGCCGCTTACAGTTTGGAGCGGATTGTGTCG-3' (reverse). The size of the expected PCR product of *SlSCP2* was 458 bp. The primers for the fusion gene of *SlSCP2* and *zeocin* were 5'-GGTACCATGGCCATGTACCGCAAAGGATTCGC-3' (forward) and 5'-GATCGGTGCGGGCCTCTT-3' (reverse). The size of the expected PCR product of *SlSCP2* and *zeocin* was 1166 bp.

RESULTS

Development of the preliminary stably transfected cell lines

By using the Trypan Blue staining and colonies scoring, 500 μg/ml of hygromycin was confirmed as the optimal concentration for resistance selection of CHO cells, in which all hygromycin-treated normal cells

Table 1: Mortality rates (%) of normal CHO cells treated with different concentrations of hygromycin.

Hygromycin (μg/ml)	1st group	2nd group	3rd group
0	20	1.3	0.8
50	62.8	74.9	81.4
100	82.4	91.1	94.1
250	0.96	99.7	98.5
500	100	100	99.7
750	100	100	99.7
1000	100	100	100

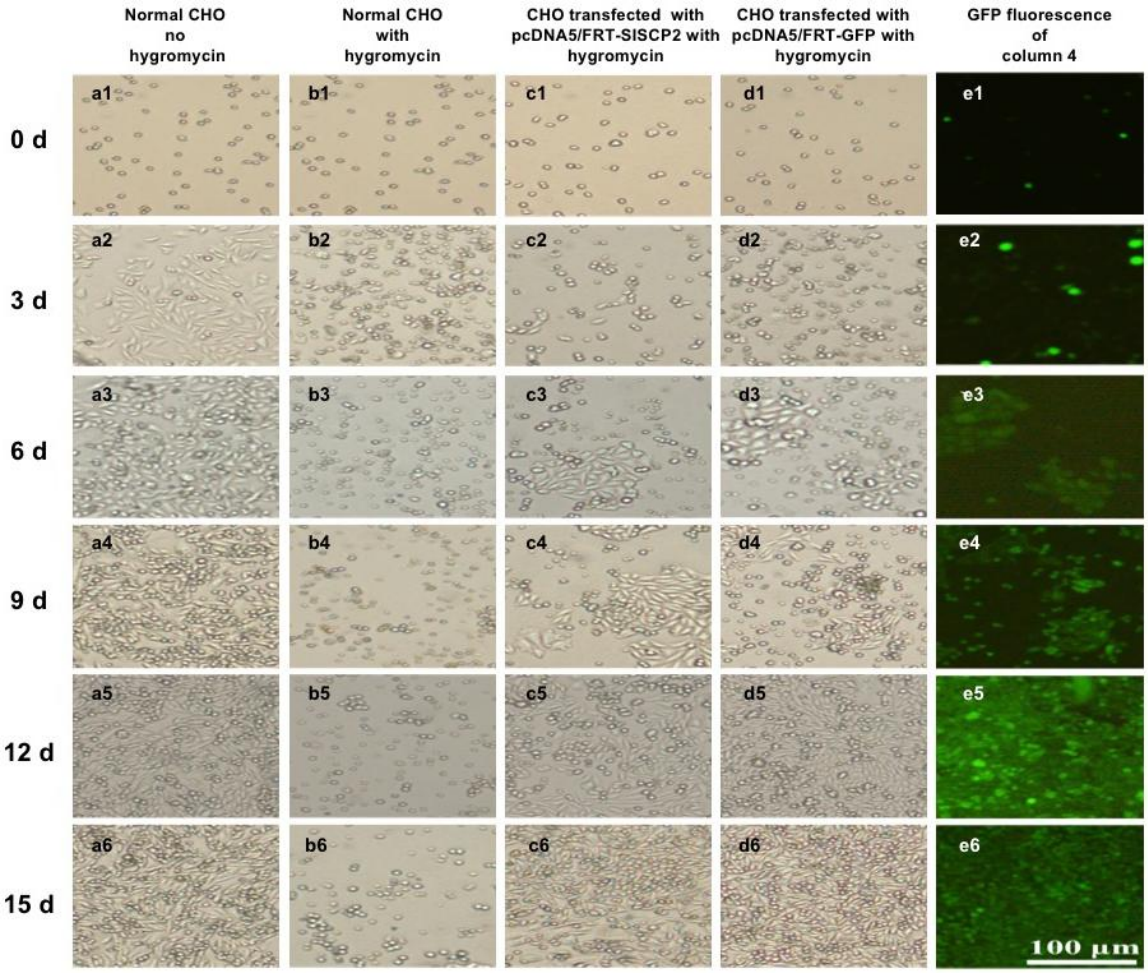

Figure 1: Growth of the normal and transfected CHO cells that were cultured in the medium in the absence or presence of 500 μg/ml Hygromycin. a1-6: untransfected CHO cells without hygromycin; b1-6: untransfected CHO cells treated with hygromycin as negative control; c1-6: CHO cells transfected with the plasmid pcDNA5/FRT-SlSCP2 with hygromycin; d1-6: CHO cells transfected with the plasmid pcDNA5/FRT-GFP with hygromycin as positive control; e1-6: the fluorescence of GFP.

died after 5 days (Table 1). The CHO cell line was transfected with pcDNA5/FRT-SlSCP2 and the control vector pcDNA5/FRT-GFP, respectively. Compared with the normal cells (Figures 1a and 2), a large number of the untransfected cells (Figures 1b and 2) and pcDNA5/FRT-SlSCP2 (Figure 1c and 2) or pcDNA5/FRT-GFP transfected cells (Figures 1d and 2) died after three days and floated in the medium when hygromycin was present at 500 μg/ml (Figure 1). The non-transfected cells started to die in the 6th day and completely died by the 15th day (Figures 1b, 3b and 6). However, the pcDNA5/FRT-SlSCP2 or pcDNA5/FRT-GFP transfected cells appeared spindle

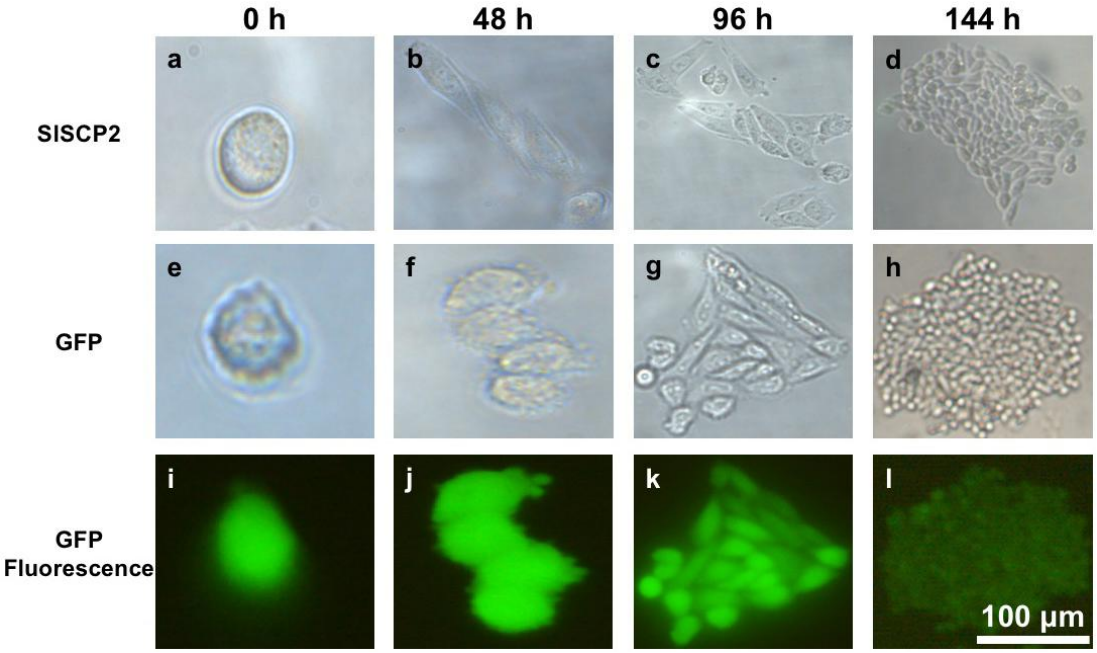

Figure 2: The selection of the single colony cells expressing SlSCP2. a-d: CHO cell transfected with the plasmid pcDNA5/FRT-SlSCP2; e-h: CHO cell transfected with the plasmid pcDNA5/FRT-GFP; i-l: the fluorescence of CHO cell transfected with the plasmid pcDNA5/FRT-GFP.

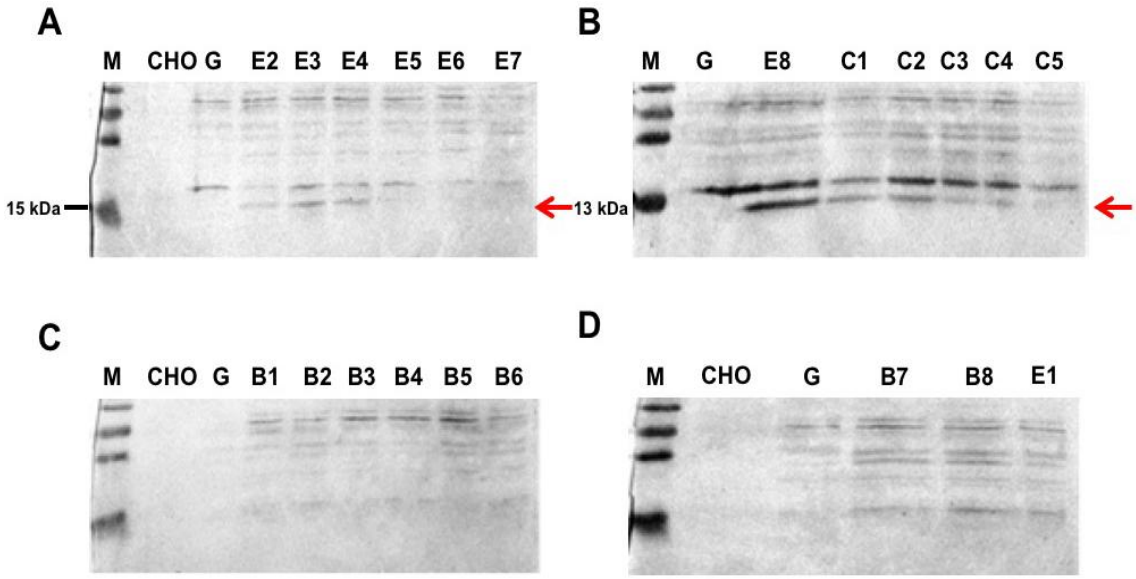

Figure 3: Western blotting analysis of the expression of SlSCP2 in the single colony cells after 4 passages in culture. CK: SlSCP2 recombinant protein in a bacterial expression system: CHO: The total proteins of CHO cells; G: The total proteins of the GFP expressing cells; B1-8, E1-8 and C1-5: The total proteins of the pcDNA5/FRT-SlSCP2 transfected monoclonal cells.

shape and aggregated into cell clusters. These cells continued division and growth and finally covered the whole culture well by the 15th day (Figures 1c, 3c, 6, d3 to d6). The fluorescence of GFP proved that the inserted GFP gene was expressed in the primary transfected cells (Figure 1e1 to e6).

Screening for mono-colonies of SlSCP2 and GFP transfected cell lines by density gradient dilution method

To obtain the mono-colonies of stably transfected cell lines, the preliminary stably transfected cell suspension was

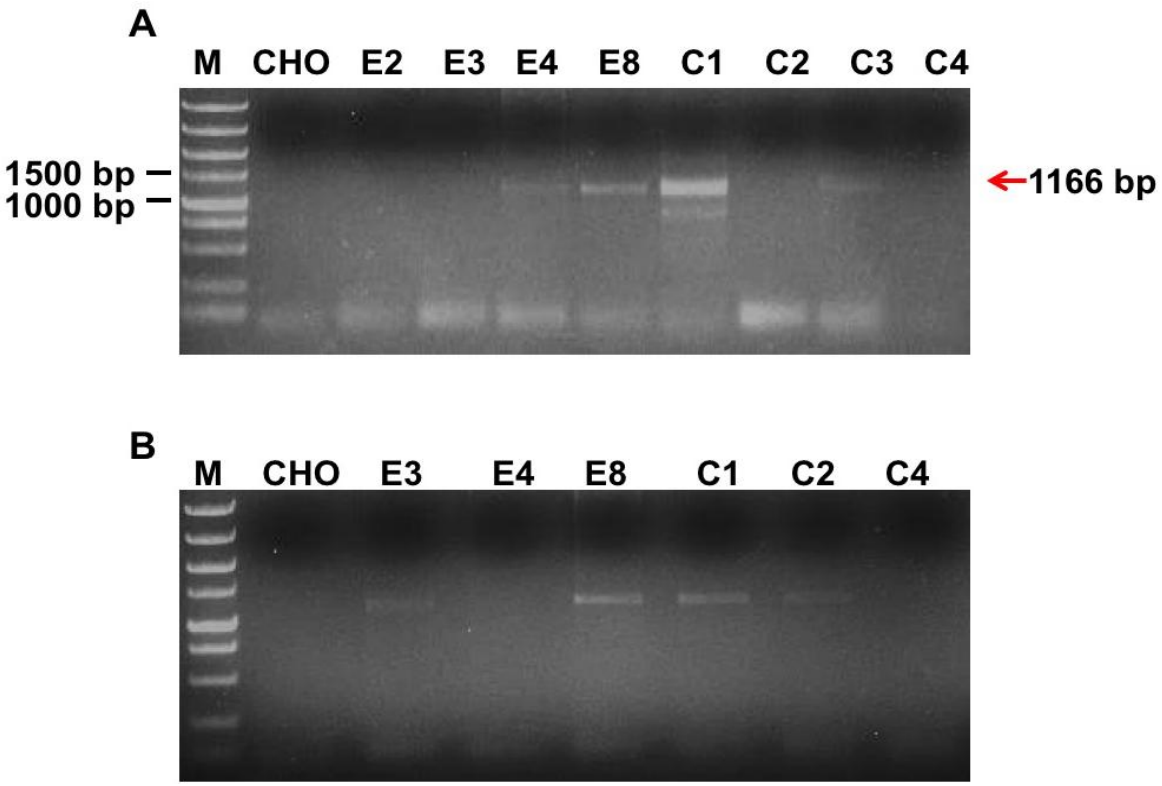

Figure 4: The genomic PCR analysis of the single colony cells after 4 (A) and 8 (B) passages in culture. CHO: The genomic DNA of the non-transfected CHO cells; E2, E3, E4, E8, C1, C2, C3, C4: The genomic DNA of the pcDNA5/FRT-SlSCP2 transfected monoclonal cells.

diluted to a concentration of 5 cells/ml. A hundred µl of the cell suspension was seeded per well in 96-well plates. In total, 31 wells that contained only a single cell were selected for subsequent culture and for target gene expression analysis with 21 lines for pcDNA5/FRT-SlSCP2 and 10 lines for GFP. These mono-colony cell lines were grown in the culture medium containing 500 µg/ml hygromycin, which was changed every three days. With the prolongation of time, the *SlSCP2* transfected mono-colonies (Figure 2a to d) and the *GFP* transfected mono-colonies (Figure 2e to h), which displayed green fluorescence (Figure 2j to l) continued to grow and divide due to the hygromycin resistance endowed by the resistant gene. These mono-colonies cells were cultured from 70 to 80% confluency and then sub-cultured for three passages in the presence of hygromycin before they were used for protein analysis of SlSCP2 and GFP expression.

Identification of monocolonal SlSCP2 and GFP transfected cell lines by Western blotting and genomic PCR

After four passages in culture, *SlSCP2* and *GFP* transfected cells were collected for Western blotting analysis. The result showed that eight (E2, E3, E4, E8, C1, C2, C3 and C4) out of 21 SlSCP2 transfected cell lines expressed 13 kDa SlSCP2 protein (Figure 3A and B), while no target protein was detected in the other 13 monocolonal cell lines (Figure 3C and D). Genomic PCR that amplified the fusion gene of *SlSCP2* and zeocin showed that only E8 and C1 of the eight cell lines had the stable integration of the target gene into the genomic DNA in the cells of both 4th generation (Figure 4A) and 8th generation (Figure 4B).

Stability analysis of stably transfected cell lines

To test whether or not the transferred gene stably stayed in the genome of the cells after high generation of passages in culture, non-transfected cells and the pcDNA5/FRT-SlSCP2 stably transfected cells after the 12th generation were treated with 500 µg/ml hygromycin and observed every 24 h. The non-transfected cells died and floated (Figure 5A a to f) with a rapid decrease in cell growth rate (Figure 5B right). The pcDNA5/FRT-SlSCP2 transfected cells continued to divide and grew (Figure 5A g to l). The growth rate of the stably transfected cell lines increased as a result of the hygromycin-resistance (Figure 5Ba). The results indicated that after 12

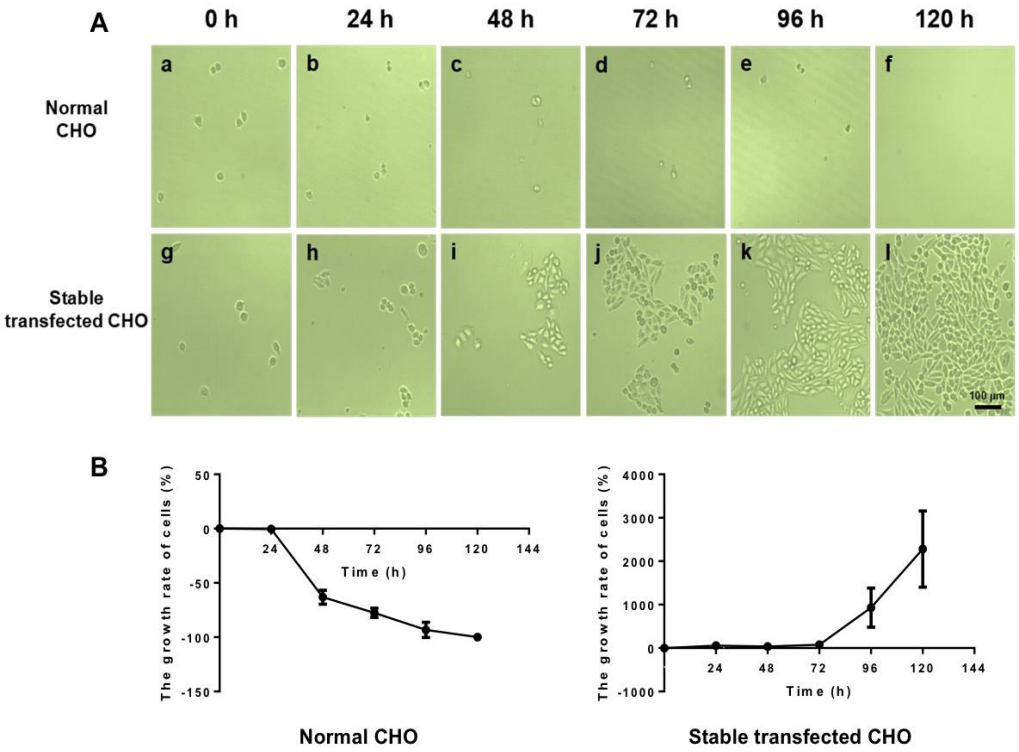

Figure 5: Analysis of growth (A) and hygromycin-resistance; (B) of the non-transfected A(a-f) and the pcDNA5/FRT-SlSCP2 transfected A(g-l) CHO cells after 12th generations of passages in culture. B left: Analysis of drug-resistance of the non-transfected CHO cells; B right: Analysis of drug-resistance of the plasmid pcDNA5/FRT-SCP2 transfected CHO cells.

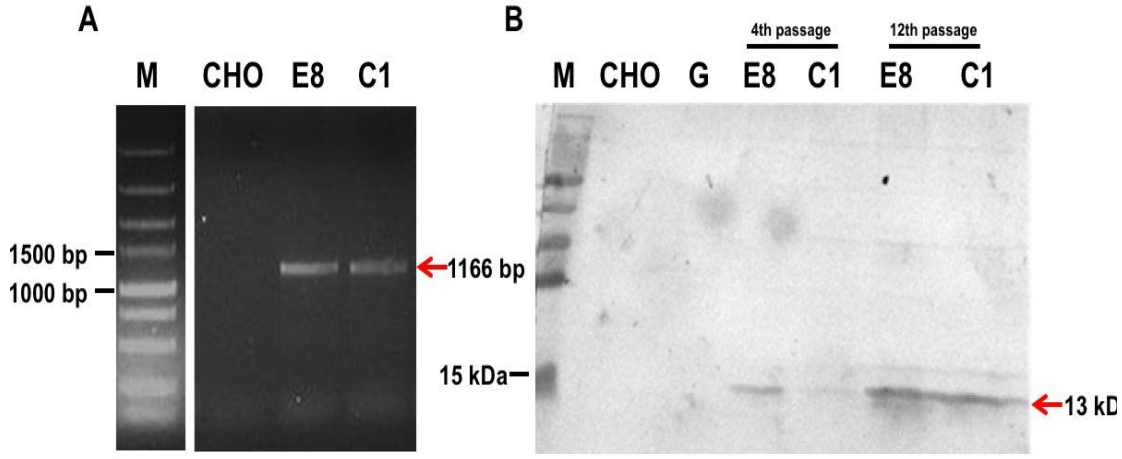

Figure 6: Genomic PCR and Western blotting analysis of the *SlSCP2* gene in monocolonal cells after 12 generations in culture. (A) Genomic PCR analysis. CHO: the non-transfected CHO cells; E8 and C1: pcDNA5/FRT-SlSCP2 transfected monocolonal cell lines; (B) Western blotting analysis. CK: SlSCP2 recombinant protein expressed in a bacterial expression system; CHO: The total proteins of CHO cells; G: The total proteins of the GFP expressing cells; E8 and C1: The total proteins of the pcDNA5/FRT-SlSCP2 transfected monocolonal cell lines.

generations, *SlSCP2* and the hygromycin genes were still stably integrated into the cell genome.

To detect whether or not the E8 and C1 mono-colony cell lines stably expressed the target protein SlSCP2, genomic PCR and Western blotting were performed and the results showed that the inserted pcDNA5/FRT-SlSCP2 could be amplified in the genome of the cells of these two lines (Figure 6A) and the SlSCP2 protein was

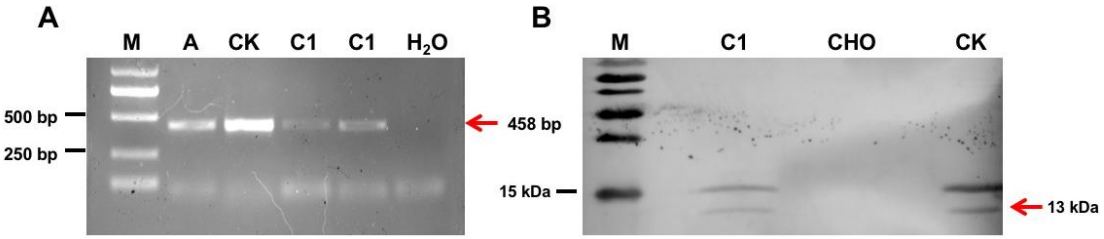

Figure 7: Genomic PCR and Western blotting analysis of the *SlSCP2* gene in monocolonal cell line C1 after 27 generations in culture. (A) PCR analysis. A: cDNA from mid-gut; CK: Bacteria which expressed *SlSCP2* gene; C1: gDNA from pcDNA5/FRT-SlSCP2 transfected monocolonal cell lines; (B) Western blotting analysis. C1: The total proteins of the pcDNA5/ FRT-SlSCP2 transfected monocolonal cell line; CHO: The total proteins of CHO cells; CK: SlSCP2 recombinant protein expressed in a bacterial expression system.

stably and highly expressed (Figure 6B) after 12 generations in culture. *SlSCP2* gene was amplified after 27 generations and SlSCP2 protein detected after 27 generations in culture (Figure 7A) for C1 monocolony cell line (Figure 7B).

DISCUSSION

Hygromycin is an aminoglycoside antibiotic, which can kill eukaryotic cells by inhibiting protein synthesis. The cell lines that are transfected with pcDNA5/FRT vector obtain the hygromycin resistance, whereas the non-transfected cell lines would were killed (Palmer et al., 1987). However, too high concentrations of hygromycin will also kill the positive monocolonal cells. On the other hand, if too low concentrations of hygromycin are used, a large number of false positive cells may survive, resulting in a low screening efficiency of transfected colonies (Bosse et al., 2013). In this study, hygromycin at the 500 μg/ml was optimized for screening SlSCP2 transfected CHO cell lines that express the *hygromycin* resistance gene.

Stably transfected cell lines have been applied to study protein function and for high throughput screening of compounds in insects. Human embryonic kidney cell line HEK293 that stably expressed *Lucilia cuprina* acetylcholinesterase was constructed and used for high-throughput screening and identification of new acetylcholinesterase inhibitors (Ilg et al., 2011). In mosquito, *Aedes aegypti,* G Protein-coupled Receptors (GPCRs) protein were stably expressed in the mammalian cell line CHO-K1 used to screen the receptor binding ligands and to identify the key amino acids for ligand binding (Lu et al., 2011).

In this study, a stably transfected CHO cell line that expressed *S. litura SlSCP2* was developed. The results of genomic PCR and Western blotting assay clearly demonstrated that the *SlSCP2* gene was integrated into the genome of CHO cell lines and stably expressed to protein, even after 27 generations of passages in culture. Out of 21 *SlSCP2* transfected cell lines selected for genomic RNA and SlSCP2 protein analysis, although all of them showed that the gene was integrated into the genome of CHO cells, but only eight lines showed the expression of the SlSCP2 protein after four or eight passages in culture. By 12 generations in culture, two lines were found to stably express the protein, while the others seem to have lost the ability of expressing the target protein (Figure 6B). These results suggest that even if a gene was integrated into the genome of CHO cells, it does not guarantee its correct and highly efficient expression into a protein, and sometimes the expression activity of the integrated gene can be lost again. How to maintain the expression activity of an integrated gene in the transfected cells should be further investigated.

The stably transfected cell lines obtained from this study provide a cell model for high-throughput screening insect growth inhibitors that target SlSCP2. Because the SlSCP2 is responsible for up-taking and transport of cholesterol in *S. litura* (Guo et al., 2009) and inhibition of the cholesterol binding with SlSCP2 would suppress the uptake and transport of cholesterol, resulting in inhibition of the insect growth and development. Thus, any small chemical compounds that compete with cholesterol for binding with SlSCP2 are the potential candidates of growth inhibitors. With this SlSCP2 transfected cell line, we can develop a cell-based and large scale screening system for chemical inhibitors of insect growth and development. In addition, this transfected CHO cell line can also be used for studying the function of SlSCP2, for example, interactions of the protein-protein, protein-membranes and protein-ligands.

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