Fusion of fluorescent protein to puromycin *N*-acetyltransferase is useful in *Drosophila* Schneider S2 cells expressing heterologous proteins

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Abstract The *Drosophila melanogaster* Schneider 2 (S2) cell line was established in 1972. Many studies have indicated that generation of recombinant proteins with S2 cells is more desirable than using other methods, since native proteins derived from S2 cells do not usually interact with those derived from mammalian cells. In order to minimize the duration for selections, we established an all-in-one single plasmid pMT-PURO, which enables to express the gene of interest as well as a selection gene “*pac*”. However, there is a weak point in the system. In order to verify the hallmark of the transformed cells, puromycin selection as well as verification of the gene of interests is still necessary. To improve this situation, we generated pMT-PURO2G and pMT-PURO2R, which enable to verify the hallmark of the transformed cells during the selections by the detection of enhanced green fluorescent protein (EGFP) or DsRED2. This new system gives reliable and reproductive results for recombinant protein synthesis and gets rid of some degree of uncertainty for the outcome of the transfection.

Keywords EGFP · DsRED2 · Puromycin · Selection · Single plasmid

Introduction

The *Drosophila melanogaster* Schneider 2 (S2) cell line was established in 1972 (Schneider 1972). These cells grow rapidly in culture at room temperature, without a need for CO₂. Many studies have indicated that generation of recombinant proteins with S2 cells is more desirable than with other methods, since native proteins derived from S2 cells do not usually interact with those derived from mammalian cells. Despite these advantages, only two markers, viz., hygromycin and blastcidin, are commercially available for establishing stable transformants (Life Technologies Japan, Tokyo, Japan).

In order to minimize the duration for selections, we established a co-expression vector for S2 cells, “pCoPURO”, that contains the *Copia* promoter (PCO), puromycin *N*-acetyl-transferase (*pac*), and the SV40 poly A sequence (pA) (Iwaki et al. 2003). After establishing the co-expressing vector, we also generated an all-in-one single plasmid pMT-PURO, which enables to express gene of interests as well as a selection gene “*pac*” (Iwaki and Castellino 2008).

These vectors are fully functional and are actually used by many researchers. However, there is a weak point in the system. In order to verify the hallmark of the transformed cells, puromycin selection as well as verification of the gene of interests is still necessary. During the selections by puromycin, a method to detect the efficiency of successful transformation is desired. To improve this situation, a new system was established. The results were summarized herein.

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Materials and methods

Construction of pMT-PURO2, pMT-PURO2G, and pMT-PURO2R

An all-in-one vector pMT-PURO (Fig. 1a) was modified by polymerase chain reaction (PCR) using two primers in order to introduce the Kozak sequence (K) upstream of the initiation codon of the *pac* gene: pMT-PURO.KozakF; 5'-ATGACCGAGTACAAG CCCACGGTG, and pMT-PURO.KozakR; 5'-GGT GGCGGCGCAAGCTATCGAATTCCTGCAGCCCCG (the Kozak sequence was underlined).

All PCR in this paper were carried out using PrimeSTAR[®] HS DNA Polymerase (TAKARA BIO, Otsu, Japan) according to manufacturer's instruction.

After the PCR, the amplicon was phosphorylated by T4 polynucleotide kinase (NEB Japan, Tokyo, Japan), and then self-ligated using Quick Ligation Kit (NEB Japan). The resulting plasmid was named pMT-PURO2 (Fig. 1b). Next PCR was carried out using following primers to prepare a backbone for insertion of enhanced green fluorescent protein (EGFP) or DsRED2 immediately downstream of *PAC* gene:

pMT-PURO2.tagF; 5'-GAGGCCCACCGACTC-TAGATCAAGC, and pMT-PURO2.tagR; 5'-CAT GGCACCGGGCTTGCGGGTCA (the underlined sequence was used as an initial codon for EGFP or DsRED2).

cDNA encoding EGFP or DsRED2 were taken from pCAG-EGFP or pCAG-DsRED (Matsuda and Cepko 2004) by PCR using the following primers sets, respectively:

For EGFP, EGFP.F; 5'-GTGAGCAAGGGCGAG-GAGCT, and EGFP.R; 5'-TTACTTGTACAGCTCG TCCATGC.

For DsRED2, DsRED2.F; 5'-GCCTCCTCCGAGA ACGTCATC, and DsRED2, R; 5'-CTACAGGAA CAGTGTTGGC.

These amplicons were phosphorylated by T4 polynucleotide kinase, and then ligated to the backbone. The resulting plasmids were named pMT-PURO2G (Fig. 1c) or pMT-PURO2R (Fig. 1d).

Construction of pMAK45, pMAK46, and pMAK47

cDNA encoding human *SERPINE1*, which is the gene for plasminogen activator inhibitor-1 (PAI-1), was

Fig. 1 Map of **a** pMT-PURO, **b** pMT-PURO2, **c** pMT-PURO2G, **d** pMT-PURO2R, **e** pMAK45, **f** pMAK46, and **g** pMAK47. *PMT* metallothionein promoter, *PCO* copia promoter, *K* Kozak sequence, *MCS* multiple cloning site, *pA* SV40 late polyadenylation signal, *AMP* ampicillin resistant gene, *puCoR* pUC origin, *PURO* puromycin *N*-acetyl-transferase (*pac*). Red and blue arrows in **a** and **b** indicate primers for inverse PCRs. (Color figure online)

taken from pcDNA3, 1-h*SERPINE1*-Wt (Iwaki et al. 2011) by PCR using following primers:

MAK45F; 5'-CTCGCTCGGGAGATCTGTGC AC CATCCCCATCCTAC (the bold part indicates *Bgl*III recognition site, and the underlined part was used for In-Fusion cloning), and MAK45R; 5'-CGA AGGGCCCTCTAGATCAGGGTTCCATCACT TG GC (the bold part indicates *Xba*I recognition site, and the underlined part was used for In-Fusion cloning).

The pMT-PURO2, pMT-PURO2G, and pMT-PURO2R were digested by the restriction enzymes, *Bgl*II (NEB Japan) and *Xba*I (NEB Japan). After the digestion, the PCR fragment containing human *SERPINE1* cDNA was cloned into them using In-Fusion cloning system (TAKARA BIO) according to the manufacturer's instruction. The resulting plasmids were named pMAK45 (Fig. 1e), pMAK46 (Fig. 1f), and pMAK47 (Fig. 1g), respectively.

Transfection of pMAK45, pMAK46, and pMAK47 to S2 cells

Non-transfected S2 cells (RIKEN BioResource Center, Tsukuba, Japan) were maintained at 25 °C in Express-Five SFM (Life Technologies, Osaka, Japan) supplemented with 10 % fetal bovine serum (FBS, Life Technologies Japan) and 1× antibiotic antimycotic solution (Sigma-Aldrich Japan, Tokyo, Japan) (complete medium). S2 cells (1×10^6) were seeded into each well of a 6-well plate containing 3 ml of complete medium, and the cells were grown for 16 h at 25 °C. A calcium phosphate transfection procedure was employed to produce stable transformants using Calcium Phosphate Transfection kit (Life Technologies Japan). Briefly, 20 µg pMAK45, pMAK46, or pMAK47 was mixed with 36 µl 2 M CaCl_2 , and then the final volume was adjusted to 300 µl with sterile water. These mixtures were slowly mixed with 300 µl 2× HEPES-buffered saline. The calcium phosphate-DNA precipitate was incubated with the cells for 16 h, after which the cells were pelleted gently for 2 min at 2,000 rpm, resuspended in 3 ml of complete medium. The cells were re-plated into the same well and incubated for 48 h. After the incubation, the cells were expanded in a 25 cm² flask in 5 ml complete medium.

Fluorescence-activated cell sorting (FACS)

The expanded cells in a 25 cm² flask were further expanded in three 175 cm² flasks until fully confluent. FACS was performed using the FACS Aria cytometer (BD, Franklin Lakes, NJ, USA). The sorted cells were cultured in an appropriate tissue culture flask and expanded as described above. This step was repeated twice.

Puromycin selection

When the expanded cells in a 25 cm² flask were at 80 % confluency, the medium were replaced with

5 ml complete medium containing 1.0 µg/ml puromycin. The cells were continuously cultured for 72–96 h. When the cells were at 80 % confluency, the medium was replaced with 5 ml complete medium containing 1.0 µg/ml puromycin. This step was repeated once.

Coomassie brilliant blue (CBB) staining and Western blot for human PAI-1

After establishing stable transformants by FACS or puromycin selection, the cells (1×10^7) were seeded onto a 6-well plate containing 3 ml complete medium with 3 µl of 500 mM CuSO_4 , and incubated for 48 h. The culture supernatants (5 µl) were electrophoresed on two sodium dodecyl sulfate polyacrylamide gels. One gel was subjected to CBB staining. The other gel was used to transfer the recombinant proteins to a PVDF membrane (Immobilized Western, Nihon Millipore, Japan) for Western blot. The membrane was exposed to a rabbit anti-PAI-1 polyclonal antibody (Cat.# SC-8979; Santa Cruz Biotechnology, Santa Cruz, CA) and a goat anti-rabbit IgG-HRP conjugate (Cell Signaling Technology Japan, Japan), and visualized with a SuperSignal West Pico kit (Thermo Fisher Scientific, Tokyo, Japan) according to the manufacturer's instructions.

Results

Construction of pMT-PURO2, pMT-PURO2G, pMT-PURO2R, pMAK45, pMAK6, and pMAK47

We were able to successfully assemble an all-in-one vector, pMT-PURO2, pMT-PURO2G, and pMT-PURO2R (Fig. 1b, c, d, respectively). All of the vital parts, such as the coding sequences for MT promoter (PMT), Copia promoter (PCO), Kozak sequence (K), poly A signal (pA), *pac* gene (PURO), DsRED2, and EGFP, were verified by DNA sequencing (data not shown). pMAK45, and pMAK46, and pMAK47 were generated by insertion of cDNA encoding human *SERPINE1* into *Bgl*II and *Xba*I sites in the multiple cloning sites (MCS) of these vectors (Fig. 1e, f, g, respectively).

Fluorescent signals from the stable transformants by FACS or puromycin selection

The S2 cells transformed by pMAK46 or pMAK47 were selected by FACS or puromycin selection. The

Fig. 2 Fluorescent signals from the stably transformed cells. **a–f** The cells transformed by pMAK46. **g–l** The cells transformed by pMAK47. **a, d, g, j** Plain images. **b, e, h, k** Fluorescence images. **c, f, i, l** Fluorescent intensity by FACS. C.S. indicates the cells selected by FACS. P.S. indicates the cells

selected by puromycin selection. An Olympus XB51 light microscope (Olympus, Tokyo, Japan) with an Olympus UPlanApo objective (magnification, 20; numerical aperture, 0.7) was used to capture these images. Scale bars indicate 100 μ m

cells transformed by pMAK46 with FACS (Fig. 2b, c) and puromycin selection (Fig. 2e, f) were almost 100 % expressing EGFP although the mean fluorescent intensity for FACS selected cells was higher. Also, the cells transformed by pMAK47 with FACS selection (Fig. 2h, i) were almost 100 % expressing DsRED2. The cells transformed by pMAK47 with puromycin selection (Fig. 2k, l) were expressing

DsRED2 about at a 90 % level. The viability and growing rates of these transformed cells were similar to those of non-transfected S2 cells (data not shown).

CBB staining and Western blot for human PAI-1

After the induction of the stable transformants with pMAK45, pMAK46, and pMAK47 by addition of

Fig. 3 **a** CBB staining and **b** Western blot for human PAI-1. The black arrows indicate human PAI-1. Lane M Molecular marker. Lane 1 The supernatant from non-transformed S2 cells. Lane 2 The supernatant from the cells transformed by pMAK45 and selected by puromycin. Lane 3 The supernatant from the cells

transformed by pMAK46 and selected by puromycin. Lane 4 The supernatant from the cells transformed by pMAK47 and selected by puromycin. Lane 5 The supernatant from the cells transformed by pMAK46 and selected by FACS. Lane 6 The supernatant from the cells transformed by pMAK47 and selected by FACS

CuSO₄, the recombinant PAI-1 was detected by CBB staining (Fig. 3a) and Western blot (Fig. 3b). The expression levels of PAI-1 in various cells were almost equivalent.

Discussion

Using pMT-PURO we have successfully expressed several human and murine proteins related to coagulation and fibrinolysis, e.g., plasminogen, urokinase-type plasminogen activator, coagulation factor XII, high molecular weight kininogen, and prekallikrein along with a large number of variants of these proteins (Iwaki and Castellino 2008). The previous method was well established to synthesize large amounts of recombinant proteins.

In order to verify the hallmark of the transformed cells, however, it was necessary to establish the cells by puromycin selection and to induce the synthesis of recombinant proteins. Hence, a method to verify the hallmark of the transformed cells during the selections was desired.

We generated pMT-PURO2G and pMT-PURO2R, which enable to verify the hallmark of the transformed cells during the selections by the detection of EGFP or

DsRED2. These fluorescent proteins were detectable as early as 16 h after the transfection. Hence, losses of valuable time due to failures of experiments such as unsuccessful transfection and reduced cell viability during transfection can be minimized. Furthermore, expressing these fluorescent proteins did not influence the viability and growth rates of the cells. Moreover, the ability to synthesize recombinant proteins by using pMT-PURO2G or pMT-PURO2R was not different to that by using pMT-PURO2. Indeed, the expression levels of human PAI-1 by pMAK46 or pMAK47 were equivalent to those by pMAK45. Although both FACS and puromycin selection were functional to establish stable transformants, puromycin selection was much easier to handle and cheaper to use. Hence, we suggest to use puromycin selection with the new system at first.

This new system gives reliable and reproductive results for recombinant protein synthesis and gets rid of some degree of uncertainty for the outcome of the transfection. The vectors pMT-PURO2, pMT-PURO2G, pMT-PURO2R, pMAK45, pMAK46, and pMAK47 will be available from Riken Bio resource center (<http://www.brc.riken.jp/lab/dna/>).

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