pDsRed-EGFPmtag-, an effective dual fluorescent reporter system for cell-based screens of premature termination codon

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Abstract A large number of inherited diseases are caused by premature termination codon (PTC) mutations that lead to the degradation of mRNA template. In this report, we developed a dual fluorescent reporter that relied the feature of fluorescent protein coding region to express a fusion protein from pDsRed-EGFPmtag-. Expression of the fusion protein from a single reporter provides a sensitive approach for high-throughput screening of cell-specific PTC events in mixed cell cultures. Results from the read-through analysis of COS7 cells carrying the nonsense mutation pDsRed-EGFPmtag-Y445X treated by PTC 124 showed EGFP transcript level was increased in the COS7 cells treated by PTC124 in a dose-dependent manner. This novel reporter system was applicable to the majority of different PTC patterns and could be used to quantify efficiency of read-through within a single cell or select cells carrying PTC.

Keywords Dual fluorescent reporter · Premature termination codon (PTC) · Read-through efficiency

Introduction

Approximately 30 % of human inherited diseases are caused by premature termination codons (PTCs) or by frame shifts that generate nonsense codons leading to the production of a truncated protein and mRNA degradation by the nonsense mediated mRNA decay (NMD) pathway (Rehwinkel et al. 2006). Considerable interest has focused on PTCs as potential targets for treatment with small molecules (Bidou et al. 2004; Linde and Kerem 2008; Zingman et al. 2007). These molecules lead to read-through, in which an amino acid is incorporated in place of the stop. Aminoglycoside antibiotics such as amikacin (Du et al. 2006), gentamicin (Barton-Davis et al. 1999), and G418 (Bedwell et al. 1997) have been shown to interfere with the mammalian ribosome, inducing the read-through of premature stop codons (PTCs). Few non-aminoglycoside molecules [such as negamycin (Arakawa et al. 2003) and PTC124 (Finkel 2010)] also have been investigated for their ability to induce PTC read-through in cellular and animal models of genetic diseases. Two high-throughput screens were performed to identify PTC124 (3-[5-(2-fluorophenyl)-[1,2,4] oxadiazol-3-yl]-benzoic acid, C₁₅H₉FN₂O₃) as a candidate for further development that can facilitate the read-through of PTCs and restore functional protein production (Welch et al. 2007). The molecule was described as effective in animal models of Duchenne muscular dystrophy (DMD) and in models of

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cystic fibrosis (CF) (Du et al. 2008). This led to human clinical trials (Kerem et al. 2008).

Monochromatic reporters require all-or-none read-through decisions for on or off expression of GFP because it is difficult to compare fluorescence intensity of different cells without an internal control. As the majority of read-through events are not regulated as all or none, read-through events must be manipulated to fit into an all-or-none output. In this study, we developed a novel approach to quantify the ratio of two fluorescent proteins expressed from a single reporter. The reporter described here contains DsRed upstream of EGFP such that DsRed and EGFP were expressed from one reading frames. Fluorescent protein DsRed that lacks stop codons fused with EGFP was used in this system. Inclusion of a multiple cloning site (MCS) region of the appropriate size located between the DsRed and EGFP reading frames. A fragment could be embedded here and expressed with the fusion. The resulting expression of DsRed and EGFP proteins provided high sensitivity and a quantitative measure of the ratio of read-through patterns. The read-through levels of non-sense mutations were analyzed in the presence or absence of PTC124. To quantify the ratio of read-through events within individual cells in the flow cytometry analysis, and identify cells expressing different read-through patterns within a mixed cell culture, the pDsRed-EGFPmtag- expression plasmid reporter system, in which translation termination codon of the DsRed was removed, was developed in this report.

Materials and methods

Plasmid construction

Figure 1a shows the construction of the dual chromatic expression vector pDsRed-EGFPmtag-. Sequences upstream of DsRed have been converted to a Kozak consensus translation initiation site to increase translation efficiency in eukaryotic cells. The multiple cloning site (MCS) was between the DsRed and the EGFP coding sequence. Fragments cloned into the MCS were expressed as fusions to the DsRed and EGFP. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3' end of the EGFP mRNA. The vector backbone contains an SV40 origin

for replication in mammalian cells expressing the SV40 T antigen. A neomycin-resistance cassette allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of the cassette confers kanamycin resistance to *E. coli*. The backbone also has a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production. The pDsRed-EGFPmtag- expression plasmid was constructed by PCR amplifying DsRed from pDsRed-N1 (Clontech, Mountain View, CA, USA) using primers that removed the DsRed translation termination codon. The sequence of primers was as followed:

DsRed-F: 5'-TATCTCGAGCGCCACCATGGTGCGCT-3';
DsRed-R: 5'-GCGCGGAATTCCTACAGGAACAGGTGGT-3'.

The DsRed open reading frame (ORF) was placed into pEGFP-N1 (Clontech). The gene, DsRed, was amplified by PCR and flanked by restriction enzymes (*XhoI* and *EcoRI*) that were unique to the final pDsRed-EGFPmtag- expression plasmid. The DsRed sequence was placed upstream of EGFP such that the ORF of DsRed was in the same frame with the downstream EGFP (Fig. 1). The reading frame was also designed such that the initiating ATG for DsRed was in frame with EGFP.

Rapid site-directed mutagenesis PCR

For nonsense mutant, three mutagenic segments, A and B, of the target gene were amplified from the plasmid DNA template by two separate PCR amplifications. Up primers carrying nonsense base were used for the A segment, whereas down primers were used for the B segment. Segment C was the mixture of segments A and B. The PCR mixture consisted of 1 µl of plasmid DNA as template (100 ng), 1 µl of each primer (Y445XF: 5'-CGAGGGCCGCCCCCTAAGAGGGCCACA-3'; Y445XR: 5'-TTAGGGGCGGCCTCGCCCTCGCCC-3'), 5 µl of dNTP mixture, 2.5 µl of 10× buffer, 15.5 µl of distilled H₂O, and 1 µl of Pfu DNA polymerase (2.5 U). The segments C were digested with restriction enzymes *DpnI*. All of the nicked vectors, which resulted from the products in segments C, were used to transform competent *E. coli* and the nicked site was fixed by the rescue system in *E. coli*.

Fig. 1 Strategy for dual chromatic expression of DsRed and EGFP and sequence determination of pDsRed-EGFPmtag- and pDsRed-EGFPmtag-Y445X. **a** Construction of the dual chromatic expression vector pDsRed-EGFPmtag-. **b** Sequence TAC in pDsRed-EGFPmtag- was obtained from stop codon TAA by rapid site-directed mutagenesis PCR. **c** PTC TAA in pDsRed-

EGFPmtag-Y445X was acquired from codon TAC by rapid site-directed mutagenesis PCR. **d** Schematic of the reporter design used here, which accommodated reading frames with and without PTC. This results in fusion synthesis when the PTC was readthrough and EGFP synthesis when it was included

Read-through efficiency analysis in cell culture

The read-through level of non-sense mutations was analyzed in the presence or absence of PTC124 (Exclusive Chemistry Ltd. Obninsk, Russia). COS7 cells (provided by Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM (HyClone, Thermo Scientific, Logan, UT, USA) with 10 % fetal calf serum (Evergreen, Hangzhou, China) and kept in a humidified atmosphere containing 5.5 % CO₂, at 37 °C. This cell line was transiently transfected using the QIAGEN Supplementary Protocol (QIAGEN, Shanghai, China) with reporter plasmid and, on the following day, cells were washed with fresh medium, with or without PTC124 supplementation. The read-through efficiency was estimated by calculating the ratio of EGFP to DsRed fluorescent intensity. For each construct, at least three independent transfection experiments were performed.

Transfection, RNA extraction and quantitative RT-PCR (qRT-PCR) analysis

Cell cultures for COS7 was prepared, maintained and transiently transfected. qRT-PCR analysis was performed using these primers:

qDsRed-F (5'-GCTCTCCAAGAACGTCATC-3');
qDsRed-R (5'-CCGCCCTTGGTCACCTTCAG-3');
qEGFP-F (5'-CAGTCCGCCCTGAGCAAAGA-3');
qEGFP-R (5'-GTCCATGCCGAGA GTGATCC-3').

Expression analysis of DsRed and EGFP in the pDsRed-EGFPmtag- and pDsRed-EGFPmtag-Y445X by fluorescence microscopy

COS7 cells were plated at a density of 1.5×10^5 cells per well on coverslips (Sangon, Shanghai, China) for transfection. The cells were transiently transfected using the QIAGEN Supplementary Protocol (QIAGEN). 48 h post-

Fig. 2 Expression of DsRed and EGFP from pDsRed-EGFPmtag- and pDsRed-EGFPmtag-Y445X. **a** Fluorescence microscopy analysis of the pDsRed-EGFPmtag- reporter plasmid. Cells in cultures expressing pDsRed-EGFPmtag- contained

consistently both DsRed and EGFP consistent. **b** pDsRed-EGFPmtag-Y445X clearly exhibited a shift towards nearly exclusive expression of EGFP and DsRed fluorescence was only minimally detected

transfection, coverslips were washed once with PBS (phosphate-buffered saline). Images were collected using channel 488 nm and channel 558 nm, respectively.

Read-through efficiency analysis by flow cytometry assay

A total of 1.5×10^5 cells were plated per six well plate, transiently transfected using the QIAGEN Supplementary Protocol (QIAGEN) with pDsRed-EGFPmtag-. Eight hours following transient transfection, cells were treated with PTC124 at concentrations between 0.3 $\mu\text{g/ml}$ up to 16 $\mu\text{g/ml}$. Then, cells were dissociated in 0.25 % trypsin (Sangon Biotech, Shanghai, China) with vigorous pipetting to completely dissociate cells 48 h after transient transfection, centrifuged at $800 \times g$ for 5 min with slow stop and resuspended in PBS for flow cytometry assay. Flow cytometry assay was performed on FC500 (Beckman Coulter), and data were analyzed using Expo V2.0

software. Statistical analysis: For each experiment, at least three independent transfection experiments were performed. Data are expressed as means $\pm$ SD ($n = 3$). *P* values less than 0.05 were considered statistically significant.

Results

Construction of pDsRed-EGFPmtag- and pDsRed-EGFPmtag-Y445X

We aimed to develop a reporter which would express EGFP protein with different fluorescent intensities and DsRed protein as an internal control. As shown in Fig. 1a, DsRed protein was expressed as fusion to the N-terminus of EGFP as they are in the same reading frame and there was no intervening stop codon. To test whether this was a viable strategy, and in particular to determine whether PTC present or not will express the expected proteins, we generated two expression

Fig. 3 Flow cytometry analysis of pDsRed-EGFPmtag- expressing cells. Cells determined to be expressing both red and green were gated in the untransfected sample. The y axis represents intensity of red fluorescence and the x axis represents the intensity of green fluorescence

plasmids for either DsRed or EGFP (Fig. 1). The sequence determination of these plasmids conformed that both of the pDsRed-EGFPmtag- and pDsRed-EGFPmtag-Y445X were constructed successfully (Fig. 1b).

Expression level analysis of DsRed and EGFP in the pDsRed-EGFPmtag- and pDsRed-EGFPmtag-Y445X reporter system

As shown in Fig. 2, cells transfected with pDsRed-EGFPmtag- expressed green and red fluorescence. Equal exposures of cells transfected with pDsRed-EGFPmtag-Y445X plasmid demonstrated that green fluorescence was not detected and red fluorescence was only minimally detected (Fig. 2b).

Read-through efficiency analysis

To determine whether this reporter was applicable to cell sorting, transiently transfected COS7 cells were trypsinized and used for flow cytometry analysis (Fig. 3). As shown in Fig. 3, compared to the negative control, the expression of fusion protein could increase in this reporter system without carrying PTC according to the design. Consistent with results from fluorescence microscopy analysis, cells transfected with the pDsRed-EGFPmtag- contained a mixture of cells expressing red and green fluorescence. We concluded that this dual fluorescent reporter provided a sensitive assay for sorting cell populations based on read-through patterns.

Then, by treating the COS7 cell with PTC 124, we investigated whether PTC 124 could restore the

Fig. 4 Effects of PTC 124 treatment on the nonsense mutation pDsRed-EGFPmtag-Y445X. COS7 cells carrying the nonsense mutation pDsRed-EGFPmtag-Y445X were treated with PTC 124 (0.3–19.2 $\mu\text{g/ml}$) for 48 h and levels of readthrough were determined by flow cytometry analysis. * $P < 0.05$ for comparisons to untreated cells

Fig. 5 qPCR analysis of PTC 124 treatment on the nonsense mutation pDsRed-EGFPmtag-Y445X. Levels of mutant mRNA were determined by quantitative PCR. The results of each experiment are expressed relative to the quantity of mRNA in the DsRed transcription as an internal control. * $P < 0.05$ for comparisons to untreated cells

production of EGFP protein from the mutated gene pDsRed-EGFPmtag-Y445X. The nonsense mutations displayed a dose-dependent response to PTC 124 (Fig. 4). We also evaluated the read-through levels for this nonsense mutation in the presence of the aminoglycoside gentamicin and G418, and the read-through levels were lower than those in the PTC 124 treatment groups.

The ability of read-through event to antagonize NMD in mammalian cells has been reported in several reports (Allamand et al. 2008; Bedwell et al. 1997). Quantitative PCR analysis of COS7 cells carrying the nonsense mutation pDsRed-EGFPmtag-Y445X treated by PTC 124 was performed. EGFP transcript level was increased in the COS7 cells treated with PTC124 in a dose-dependent manner. Moreover, as shown in Fig. 5, EGFP transcript level was not affected by PTC124 in the COS7 cells carrying the wild-type pDsRed-EGFPmtag- gene, which demonstrated this dual fluorescent reporter system could work with the presence of the nonsense mutation.

Discussion

Several recent studies demonstrated that some read-through-stimulating molecules could induce the genes carrying a PTC to express the full-length proteins (Rowe and Clancy 2009). This approach has been applied to several cell culture models of different diseases, demonstrating the potential value of this strategy for treating patients with genetic disorders linked to the presence of

a non-sense mutation. Incubation of the cells transfected with a reporter such as a cell-based firefly luciferase (FLuc) results in the up-regulation of FLuc activity in response to PTC suppressor. In universal methods, the last step of the experiment is to dilute the medium with lysis buffer and add a very high concentration of the FLuc substrate luciferin. The luciferin essentially outcompetes the reversible inhibitor and produces a luminescent signal which is mistakenly attributed to facilitated read-through of the PTC mutation. In this study, this dual fluorescent reporter assay, and the positive, read-through data generated directly with incubated cells could reveal that PTC suppressor may exhibit genuine read-through activity in spite of its interference in the FLuc assay system. This is the outstanding highlight of the design in this report. This reporter is directly applicable for high-throughput analyses of read-through event. Expression of different fluorescent proteins from a single reporter has two advantages compared to a monochromatic read-out or the use of two separate constructs to perform a bifluorescent read-out: (1) this system has a high sensitive read-out which allows quantification of the complete output of a gene, so it is particularly useful for quantitative assessment of read-through efficiency within single cells. (2) The single dichromatic reporter removes the variability associated with co-expression of two separate reporters that express different fluorescent proteins (Fig. 1d).

Several factors determine the efficacy of read-through promoting molecules. Various stop codons are

suppressed with different efficiencies (UGA > UAG > UAA), and the efficacy of suppression depends in part on the identity of the +4 nucleotide immediately downstream from the stop codon (C > U > A > G) (Howard et al. 2000; Manuvakhova et al. 2000). So, different PTC patterns within the MCS region between the DsRed and EGFP reading frames could be measured sensitively.

The neomycin-resistance cassette in this reporter allows stably transfected eukaryotic cells to be selected by using G418. So, this reporter can be used firstly to quantify the ratio of read-through events within individual cells, for flow cytometry analysis and is applicable to fluorescence activated cell sorting (FACS) analysis. Moreover, it can be used to identify cells expressing different PTC patterns within a mixed cell culture.

In conclusion, it is a novel reporter system that contains two fluorescent proteins from different sources: EGFP from the *Aequorea* genus of jelly fish and DsRed from the *Discosoma* genus of coral. In addition to providing the opportunity to generate a bichromatic read-out, it is likely that this unusual property has biological implications with regard to how fluorescent proteins or adjacent or overlapping genes are expressed and regulated. The ability to perform quantitative single-cell analysis of PTC read-through and high-throughput screens will enhance the progress toward understanding NMD and identifying compounds that reverse pathogenic PTC defects.

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