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Fine tuning the LightOn light-switchable transgene expression system

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ABSTRACT

Spatiotemporal control of transgene expression in living cells provides new opportunities for the characterization of gene function in complex biological processes. We previously reported a synthetic, light-switchable transgene expression system called LightOn that can be used to control gene expression using blue light. In the present study, we modified the different promoter segments of the light switchable transcription factor GAVPO and the target gene, and assayed their effects on protein expression under dark or light conditions. The results showed that the LightOn system maintained its high on/off ratio under most modifications, but its induction efficiency and background gene expression level can be fine-tuned by modifying the core promoter, the UAS_G sequence number, the length of the spacer between UAS_G and the core promoter of the target protein, and the expression level of the GAVPO transcription factor. Thus, the LightOn gene expression system can be adapted to a large range of applications according to the requirements of the background and the induced gene expression.

1. Introduction

Precise spatiotemporal control of transgene expression is an indispensable tool for functional gene analysis and gene therapy. During the past two decades, several gene expression systems have been developed to control gene expression in mammalian cells using small molecules as inducers, including the TET system [1] and the GeneSwitch system [2]. These tools are now widely used in experimental biology; however, chemical inducers have relatively poor spatiotemporal resolution, they lack tissue specificity, and they are difficult to remove from live systems, thereby limiting their use in laboratory studies and industrial manufacturing.

In recent years, optogenetics has become a booming field by combining the use of light and genetically encoded light-sensitive proteins to control the behavior of living cells and organisms [3–9]. A light-switchable gene expression system can be the most promising tool for precisely controlling spatiotemporal gene expression in multicellular organisms. Several efforts have controlled gene expression using light based on the two-hybrid principle in yeast or mammalian cells, utilizing the light-induced protein interaction between *Arabidopsis thaliana* phytochrome B and PIF3 [10,11], FKF1 and GIGANTEA [7], or CIB1 and CRY2 [12]. Red/infrared and blue light were used to induce gene expression by activating phytochrome, LOV, or CRY light sensitive domains. More recently, a UVB-inducible mammalian gene control system that uses the hetero-dimerization properties of *A. thaliana* UVB receptor UVR8 and COP1 under UV illumination has been reported [13]. Ye et al. reported a synthetic signaling cascade that enables light-inducible transgene expression in vitro and in vivo [14], based on rewiring the blue light – induced, melanopsin-mediated intracellular Ca^{2+} surge to activate target gene expression via complex signal cascades.

We previously reported the simple and robust LightOn light-switchable transgene system. This system utilizes a single, synthetic light-sensitive transcription factor, GAVPO, which homodimerizes and binds to its specific promoter upon exposure to blue light and subsequently initiates the transcription of target genes. The LightOn system has several advantages over existing regulated gene expression systems that function in mammalian cells: (i) the system is simple, consisting only of one component; (ii) has low background; (iii) has high induction efficiency; (iv) has fast kinetics; (vi) has good reversibility; (vii) does not interfere with cell signaling; and (viii) it precisely controls gene expression with high spatiotemporal resolution. To further characterize the LightOn system, we modified the different promoter segments of the light switchable transcription factor GAVPO and the reporter gene, and expanded the performance of the LightOn system in terms of induction efficiency and background gene expression level. These tests provide further insights into LightOn system, enabling its wider application in biological research.

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2. Materials and methods

2.1. Plasmid constructs

The pGAVPO regulator plasmid containing the light-switchable transcription factor GAVPO and the reporter plasmid pU5-Gluc using Gaussia luciferase as the reporter gene were constructed as previously described [15]. The SV40 and PGK promoters from pCDNA3.1 hygro(+)(Invitrogen) and pMSCVpuro(Clontech) were cloned (AseI/Eco47III) into pGAVPO to obtain pSV40-GAVPO and pPGK-GAVPO, respectively. The CAG promoter was cleaved from pGAG-IRES-EGFP and inserted (SpeI/XhoI) into pGAVPO to create pCAG-GAVPO. Site-directed mutagenesis was performed according to the MutanBEST protocol (Takara) to generate pU5-Gluc reporter plasmids with spacer of different lengths between UAS_G (Gal4 recognition/biding sites) and the core promoter. The SV40 core promoter was amplified from pCDNA3.1 hygro(+) and inserted (HindIII/NheI) into pU5-Gluc to generate pU5S-Gluc reporter plasmid. The Hsp70, TK, and CMV core promoters were synthesized by Shanghai Generay Biotech Co. Ltd., and then ligated into (HindIII/NheI) pU5-Gluc to obtain pU5H-Gluc, pU5T-Gluc, and pU5C-Gluc, respectively.

2.2. Cell culture and blue light irradiation

HEK293, MCF-7, H1299, and PC-3 cells were cultured in high-glucose DMEM (GIBCO) supplemented with 10% FCS, 1% penicillin, and streptomycin (Invitrogen). The cells were plated in antibiotic-free high-glucose DMEM supplemented with 10% FCS at 14 h before transfection. We used equal amounts (0.1 μg) of the pGAVPO and reporter vectors with 0.6 μl of Lipofectamine 2000 for each well of the 48-well plate according to the manufacturer's protocol. The transfected cells were then covered with aluminum foil and kept in the dark for 10 h, and then they were illuminated with 0.84 W m^{-2} blue light from below using an LED lamp (460 nm peak) or maintained in darkness for 22 h before measurement. The LED lamps were controlled by a relay to adjust the overall intensity of blue light.

2.3. Measuring Gluc activity

The chemiluminesence of the cell samples was measured using a synergy 2 multi-mode microplate reader (Bio Tek). The secreted Gluc activity of the cell culture supernatants was assayed using a BioLux® Gaussia Luciferase Assay Kit (NEB) according to the manufacturer's protocol.

2.4. Western blot analysis

Western blot analysis was carried out using previously described procedures [16]. Briefly, equal amounts of the total lysate protein were electrophoresed on 12% SDS–PAGE gel, and then transferred onto polyvinylidene fluoride (PVDF) membranes (PALL) using an electroblotter. After blocking with 0.5% casein, the membranes were probed with mouse anti-flag (1:1000; Sigma–Aldrich) or mouse anti-β-actin (1:40,000; Sigma–Aldrich) antibodies. Subsequently, the cells were treated with horseradish peroxidase (HRP)-labeled secondary antibodies (1:10,000; Jackson ImmunoResearch). Immunoreactivity was detected using a BM Chemiluminescence Blotting kit (Roche Diagnostics) according to the manufacturer's protocol on a Kodak In-Vivo Multispectral System FX (Carestream Health).

3. Results

3.1. Effects of different core promoters on the target gene expression

Transcription is a critical component of gene expression. Eukaryotic promoters generally contain a core promoter near the transcription initiation site and one or more enhancer elements that may be located more distantly [17]. The core promoter directs high amounts of transcription via RNA polymerase II in metazoans [18]. In the pU5 vector of the LightOn system, the target gene is driven by a promoter consisting of Gal4 binding sites and the core promoter of the E1b (Fig. 1A). In this study, we replaced the E1b core promoter in the pU5-Gluc reporter vector with core promoters from the Hsp70, SV40, TK, and CMV to obtain the pU5H-Gluc, pU5S-Gluc, pU5T-Gluc, and pU5C-Gluc vectors. The vectors were introduced into the HEK293 cell line together with pGAVPO. Light induced gene expression was studied by measuring Gluc-derived chemiluminesence. The results showed that the core promoters significantly affected gene induction efficiency and background gene expression. pU5C-Gluc showed a twofold increase in induction efficiency relative to pU5-Gluc, similar to human cytomegalovirus immediate early promoter (CMV), but its background under dark conditions was significantly higher, resulting in a lower induction ratio. The reporter vectors with the SV40 and the TK core promoters showed similar induction efficiencies to the reporter vector with the E1b core promoter, but with significantly higher background gene expression levels. Although Hsp70 had less than 10% the induction efficiency of E1b, its background was extremely low; thus it may be useful when background expression should be minimal, for instance, in functional studies involving extremely potent or toxic proteins (Fig. 1B).

3.2. Alteration of UAS_G number and the length of the spacer between UAS_G and the core promoter

To determine the optimum promoter configuration, we altered the number of UAS_G and the length of the spacer between UAS_G and the core promoter. The five UAS_G in the pU5-Gluc were reduced to four, three, two, or one to create pU4-Gluc, pU3-Gluc, pU2-Gluc, and pU1-Gluc, respectively. Although the performance of pU4-Gluc and pU3-Gluc was similar to the original pU5-Gluc vector, further reducing the UAS_G number to two or one significantly reduced the induction efficiency under light and the background gene expression in the dark. Leaky expression was almost undetectable for pU1-Gluc, which indicates very stringent control of gene expression (Fig. 1C). To determine whether the length of the spacer between UAS_G and the core promoter affects the performance of light-switchable target gene expression, we extended the spacer length by introducing different lengths of randomly selected DNA bases. The result showed decreased induction of the Gluc gene with increasing spacer length. The maximal induction ratio was observed when the spacer was 30 bp (Fig. 1D). These results show that the promoter configuration of the target gene affects the performance of the LightOn system.

3.3. Optimization of the reporter plasmid to pGAVPO mass ratio

To study the effects of different mass ratios of the response plasmid to the pGAVPO on light-switchable gene expression, we used 0.2 μg of the reporter plasmid with varying amounts of pGAVPO to obtain different mass ratios (1:0, 1000:1, 400:1, 200:1, 100:1, 40:1, 20:1, 10:1, 4:1 2:1, 1:1), and maintained the total amount of DNA at 0.4 μg/well with the pCAT vector. Maximum induction of the Gluc gene was observed at a mass ratio of 2:1, whereas maximum induction was achieved at a mass ratio of 10:1, reaching

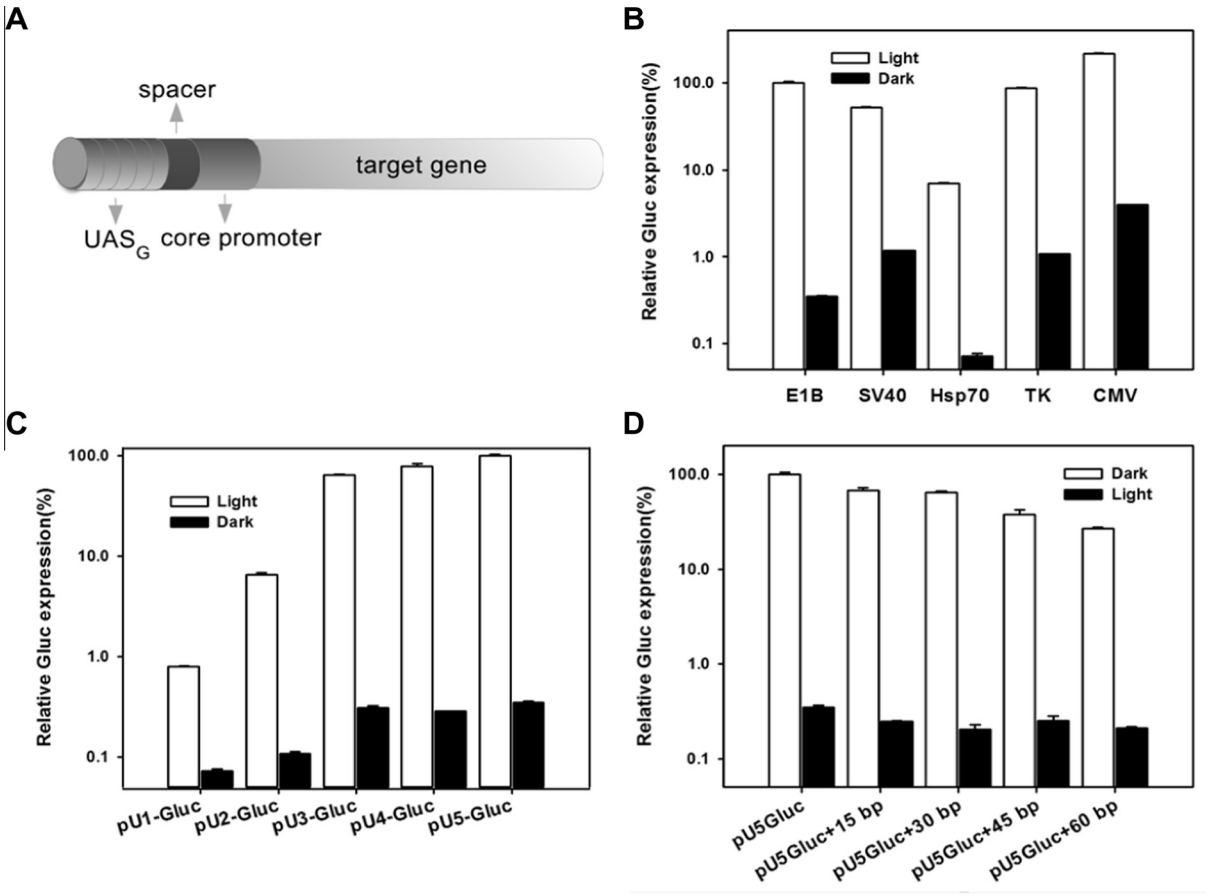

Fig. 1. Modification of the promoter segments of the target gene on reporter plasmid of the LightOn system. (A) Schematic of the reporter plasmid consisting of Gal4 recognition/biding sites (UAS$_G$), core promoter, length of the spacer between UAS$_G$ and the core promoter, and the target gene. Gluc reporter gene expression under light and dark conditions for reporter plasmids with different core promoters (B), different numbers of UAS$_G$ (C), and different spacer lengths (D). The error bars represent the standard deviations of four samples.

almost 1000-fold. Background gene expression significantly decreased and maintained extremely low almost the same with cells without pGAVPO transfection when the mass ratio was more than 20:1 (Fig. 2). Reduced transfection of the pGAVPO plasmid resulted in less expression of light-switchable regulator GAVPO. Thus, GAVPO has an important role in light-switchable gene expression, and increased GAVPO expression may increase both the induction efficiency and the background expression of the target gene.

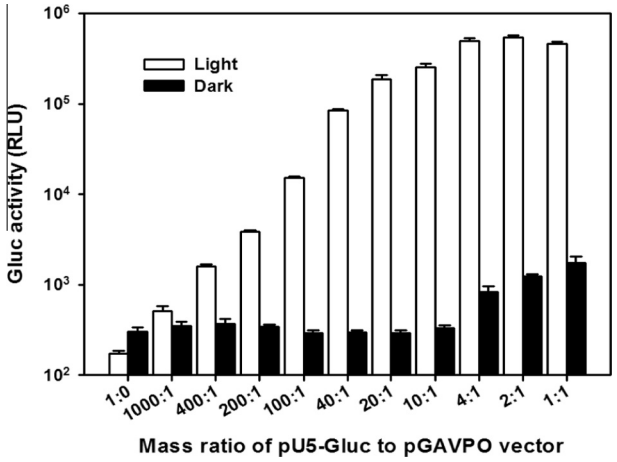

Fig. 2. Effects of different reporter plasmid to pGAVPO mass ratios on the light-induced gene expression. A constant amount of the pU5-Gluc reporter was cotransfected with varying amounts of pGAVPO into HEK293 cells. Gluc expression under light and dark conditions was determined. Cells that only obtained pU5-Gluc (1:0) transfection were used as the negative control. The error bars represent the standard deviations of four samples.

3.4. Evaluation of SV40, PGK, and CAG promoters for driving GAVPO expression on the regulator plasmid

To further validate the role of light-switchable regulator GAVPO in the light-switchable gene expression, we utilized different promoters to drive the GAVPO. The CMV expression promoter in the pGAVPO plasmid was replaced with SV40, PGK, and CAG to create pSV40-GAVPO, pPGK-GAVPO, and pCAG-GAVPO, respectively. The vectors were cotransfected with pU5-Gluc into HEK293, MCF-7, H1299, and PC-3 cells. SV40-, PGK-, and CAG-driven GAVPO expression exhibited lower induction levels than CMV-driven expression in most of the cell lines; only the CAG-driven GAVPO expression was highly effective in stimulating higher induction than CMV-driven expression in MCF-7 cells. However, SV40- and PGK-driven GAVPO expression had extremely low background expression in darkness, which can be used to tightly control the expression of toxic genes (Fig. 3A–D). Western blot analysis was carried out to detect the GAVPO expression levels driven by these promoters in HEK293 cells. Higher GAVPO expression resulted in higher induction efficiencies and higher background expression (Fig. 4), consistent with the expression levels under different reporter plasmid to pGAVPO mass ratios , which is probably because high GAVPO expression forms homodimers that do not require blue light to initiate target gene transcription. Therefore, the performance of LightOn system is dependent on the GAVPO expression level.

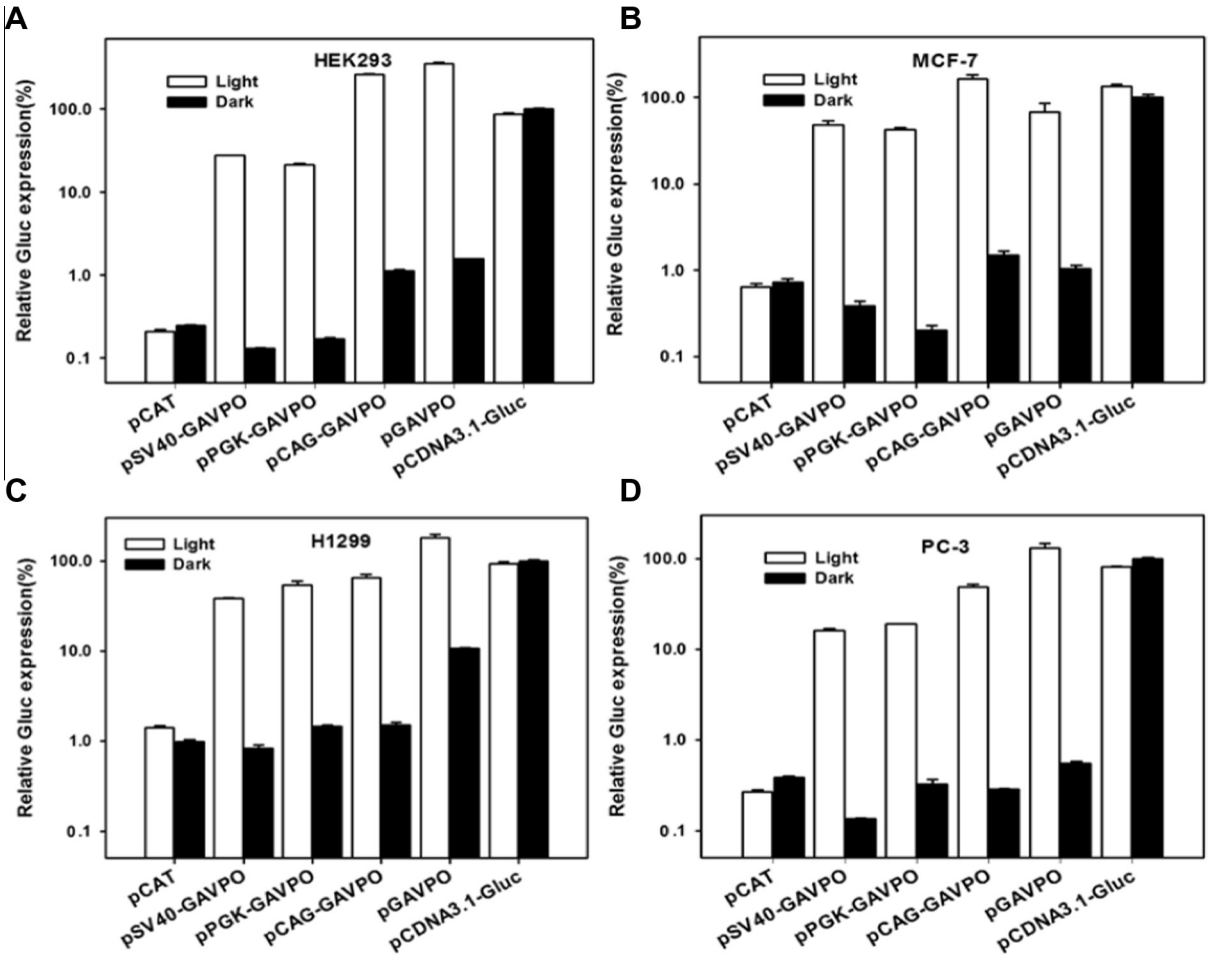

Fig. 3. Effects of SV40, PGK, and CAG promoter–driven GAVPO expression on light-induced target gene expression in different cell lines. pSV40-GAVPO, pPGK-GAVPO, pCAG-GAVPO, and pGAVPO were co-transfected with pU5-Gluc into HEK293 (A), MCF-7 (B), H1299 (C), and PC-3 (D) cells. Gluc expression was determined under light and dark conditions. Cells transfected with pCAT was used as the negative control and cells transfected with pU5-Gluc or single pCDNA3.1-Gluc were used as the positive control. The error bars represent the standard deviations of four samples.

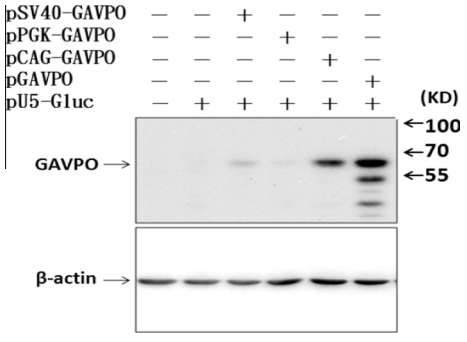

Fig. 4. Western blot analysis of GAVPO expression driven by different promoters. The regulator plasmids containing light-switchable regulator GAVPO driven by different promoters were cotransfected with the pU5-Gluc reporter plasmid into HEK293 cells. Protein lysates were electrophoresed on SDS–polyacrylamide gels and blotted with anti-flag and anti-β-actin antibodies. The cells transfected with no plasmid or the pU5-Gluc plasmid were used as the negative control.

4. Discussion

Light is an ideal inducer because it is widely available, nontoxic, and easy to manipulate. A simple and robust gene expression system that overcomes the drawbacks of traditional chemical-induced gene expression is always needed. We built a light-switchable transgene system called LightOn; it has low leaky expression, is noncytotoxic, and allows high induction with reasonably fast kinetics and good reversibility, as well as allows spatiotemporally control of gene expression. The modifications for the LightOn system in this study include the core promoter, the number of UAS_G sequences, length of the spacer separating UAS_G from the core promoter of the target protein, and the expression levels of the GAVPO transcription factor. The LightOn system with these modifications achieved maximum induction efficiency, high induction ratios, or minimum background expression. Using the CMV core promoter, shorter spacers between UAS_G and the core promoter, a greater number of UAS_G, and higher expression of the light-switchable regulator GAVPO enable higher target gene expression levels. Reporter plasmids with the PGK core promoter, one UAS_G, or a spacer length of 30 bp resulted in extremely low background expression, allowing tight control of gene expression. The induction ratio reached almost 1000-fold at a reporter vector to pGAVPO mass ratio of 10:1. Overall, this study demonstrated that the LightOn system can be conveniently adapted to applications requiring a variety of gene induction efficiencies and leaky gene expression in the dark.

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