Synthetic far-red light-mediated CRISPR-dCas9 device for inducing functional neuronal differentiation

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The ability to control the activity of CRISPR-dCas9 with precise spatiotemporal resolution will enable tight genome regulation of user-defined endogenous genes for studying the dynamics of transcriptional regulation. Optogenetic devices with minimal phototoxicity and the capacity for deep tissue penetration are extremely useful for precise spatiotemporal control of cellular behavior and for future clinic translational research. Therefore, capitalizing on synthetic biology and optogenetic design principles, we engineered a far-red light (FRL)-activated CRISPR-dCas9 effector (FACE) device that induces transcription of exogenous or endogenous genes in the presence of FRL stimulation. This versatile system provides a robust and convenient method for precise spatiotemporal control of endogenous gene expression and also has been demonstrated to mediate targetted epigenetic modulation, which can be utilized to efficiently promote differentiation of induced pluripotent stem cells into functional neurons by up-regulating a single neural transcription factor, NEUROG2. This FACE system might facilitate genetic/epigenetic reprogramming in basic biological research and regenerative medicine for future biomedical applications.

Significance

We have developed an optogenetic far-red light (FRL)-activated CRISPR-dCas9 system (FACE) that is orthogonal, fine-tunable, and reversible, and can achieve exogenous or endogenous gene regulation profiles under stimulation with FRL, with deep tissue penetration capacity, low brightness, short illumination time, and negligible phototoxicity. The FACE device is biocompatible and meets the criteria for safe medical application in humans, providing a robust differentiation strategy for mass production of functional neural cells from induced pluripotent stem cells simply by utilizing a beam of FRL. This optogenetic device has expanded the optogenetic toolkit for precise mammalian genome engineering in many areas of basic and translational research that require precise spatiotemporal control of cellular behavior, which may in turn boost the clinical progress of optogenetic-based precision therapy.

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can induce differentiation of neural-lineage cells from induced pluripotent stem cells (iPSCs). This system thus offers a versatile tool for many diverse applications that require dynamic regulation of gene expression or targeted epigenetic modifications, including the development of therapeutic interventions and the induction of cell differentiation.

**Results**

**Design and Optimization of a FACE System.** To create an efficient CRISPR-dCas9–based photoactivatable transcription system with low phototoxicity and deep tissue penetrative capacity, we constructed a FACE system (Fig. 1A–C) based on a fully orthogonal FRL-triggered optogenetic system (22) in which the BphS is an FRL-activated c-di-GMP synthase (26, 27) and the transcription factor BldD is derived from *Streptomyces coelicolor* (28, 29) to achieve dynamic regulation of gene expression. The FRL-dependent hybrid transactivator p65–VP64-NLS–BldD could dimerize in the presence of c-di-GMP and bind to its chimeric promoter pFRL to initiate downstream gene expression (Fig. 1A). These previous studies demonstrated that the synergistic activator mediator (SAM) system can be developed to enhance the efficiency of gene activation by engineering the sgRNA bearing MS2 RNA aptamers (23). By fusing MS2 proteins with various activators such as the 65-kDa transactivator subunit of NF-κB (p65) and heat shock factor 1 (HSF1) transactivation domains, sgRNA-mediated recruitment of a MS2–p65–HSF1 complex could improve the activation efficiency of dCas9-targeted endogenous genes. Therefore, we hypothesize that optical control of MS2-mediated recruitment of transcriptional activators might enable more potent transcriptional activation.

We first generated optogenetic transcriptional activation systems containing different variants of transactivators (FGTAx) by assembling MS2 fused to P65, VP64–p65–Rta (VPR), (12) or to HSF1 (Fig. 1B) recruited by sgRNAs bearing MS2 RNA aptamers, so as to assess the induction potency of genes targeted by dCas9–FGTAs. To obtain a robust light-inducible transcription system, we have constructed different transactivators (FGTAx) (Fig. 1B) and several FRL-responsive chimeric promoter variants (pFRLx) (Fig. 1C), which were assembled into various configurations. Subsequently, we tested and identified which combination induces reporter gene expression most efficiently. We found that a combination of pFRLx and FGTA1 [pFRLx–FGTA1–pA (pGY48)] resulted in the most optimal FRL-triggered exogenous transcriptional responses (Fig. 1D–H). Nevertheless, this combination did not exhibit very strong activation of endogenous achaete-scute homolog 1 (*ASCL1*) gene expression (∼38-fold) in HEK293 cells (SI Appendix, Fig. S1). Hence, we speculate that the fusion transactivator FGTA1 (MS2–VP64) is not potent enough to up-regulate the required levels of endogenous gene expression, whereas the transcriptional activator FGTA4 (MS2–p65–HSF1) displayed significant activation potential (Fig. 1D–H).

To improve robustness of the system, we further optimized the FACE system by testing the capacity of different variants of BldD-responsive promoters to up-regulate different amounts of the transactivator FGTA4 with different FRL-specific promoters (pFRLx) (Fig. 1 I–K). We found that HEK293 cells cotransfected with pWS46 (pFCMV–BphS–p2A–YhjH–pA), pGY32 (pFCMV–pFIRA3–pA), pS46 (pFCMV–dCas9–pA), pGY102 (pFCMV–FGLTA4–pA), pWS137 (pU6–sgRNA1 (pFCACE–pA)), and pWS107 (pU6–human placental secreted alkaline phosphatase (SEAP)–pA) exhibited the highest fold FRL-triggered exogenous transgene activation (Fig. 1K). In addition, this configuration could efficiently activate endogenous gene expression (Fig. 1L).

**Characterization of the Optimized FACE System.** The detailed performance of the FACE system was further characterized. To assess the impact of FRL (730 nm) and blue light (465 nm) on the metabolic integrity and viability of mammalian cells, HEK293 cells were transfected with pSEAP2-control and then were exposed to light for 48 h, followed by quantification of SEAP expression levels and cell viability (SI Appendix, Fig. S2A and B). The control experiment data demonstrated that FRL is minimally phototoxic to mammalian cells compared with blue light (SI Appendix, Fig. S2A and B). The different wavelengths of light-triggered transgene expression of the FACE system suggest that this optogenetic tool exhibited the best SEAP induction ratios under FRL (730 nm) illumination, thus indicating the chromatic specificity of the FACE device (SI Appendix, Fig. S3). We further characterized the detailed performance of the transgene expression kinetics of the FACE device. The data showed that the transgene expression triggered by FRL was illumination intensity- and exposure time-dependent (Fig. 2A and B). We then transduced the FACE device into different cell lines and found that it was functional in a number of mammalian cell types (Fig. 2C), demonstrating the applicability and compatibility of this photoactivatable transcription system in a broad range of applications. The variable transcriptional performance of the FACE device in different mammalian cell lines could probably be due to the different transfection efficiencies as well as potential interactions with endogenous cell components of different cell lines. Moreover, the FACE system was demonstrated to exhibit fully reversible transcription activation kinetics (Fig. 2D) and precise spatiotemporal transgene activation (Fig. 2E and F).

**Photoactivation of User-Defined Endogenous Genes with the FACE System.** To test whether the FACE system could control endogenous gene activation, we activated the endogenous gene target by delivering the FACE system with a group of two sgRNAs that target the promoter region of the human *ASCL1* gene into HEK293 cells. We then used qRT-PCR to assess the activation of *ASCL1* gene after 6 h of FRL illumination or in the dark and found that *ASCL1* was successfully up-regulated using each sgRNA, with significant further enhancement upon using two mixed sgRNAs compared with a single sgRNA (SI Appendix, Fig. S4). It was notable that mixed sgRNAs transfection did not affect *ASCL1* expression in the dark but resulted in high levels of induction by FRL. These results further demonstrated that our FACE system could induce endogenous gene expression by FRL and produce a high level of transcriptional activation with mixed sgRNAs. The detailed performance of the endogenous gene activation kinetics using the FACE system was further characterized. The data showed that the endogenous *ASCL1* up-regulation triggered by FRL was illumination intensity- and exposure time-dependent (Fig. 3A and B). We further confirmed that the FACE system was able to robustly activate endogenous *ASCL1* expression in different mammalian cell lines (Fig. 3C), further indicating that the FACE system has broad utility for a wide range of applications.

In addition to gaining gene activation, it is also important to have the capacity to regulate gene expression reversibly in some biological processes that exhibit temporal gene-expression patterns. We therefore tested whether endogenous gene activation by the FACE system is reversible by investigating the switch-on and switch-off kinetics of FRL-induced *ASCL1* up-regulation by the FACE device (Fig. 3D). The transfected cells were illuminated with FRL for the first 20 min, and the *ASCL1* mRNA level was sustained up-regulated in the following 12 h but subsequently decreased to baseline level within 24 h. When the same batch of cells was exposed to FRL for another 20 min after 48 h of incubation, the *ASCL1* mRNA level could be up-regulated again (Fig. 3D). Collectively, these results demonstrated that the FACE system could reversibly activate endogenous gene expression.

Additionally, we sought to investigate whether this system could be applied to induce multiple endogenous gene transcription. We therefore generated four pairs of sgRNAs with each pair targeting the promoters of titin (*TTN*), interleukin 1 receptor antagonist (IL1RAN) (21), and interleukin 1 receptor antagonist (IL1RAN) (21), 585.0x783.0
(IL1RN), ASCL1, and rhox homeobox family member 2 (RHOXF2) and tested the FRL-induced endogenous gene transcription system in HEK293 cells. The qRT-PCR results showed that the FACE system could achieve optogenetic control of multiplexed endogenous gene transcription in the genome without affecting the others (Fig. 3E), thus demonstrating simultaneous activation of user-defined endogenous gene(s) of interest with the FACE system.

Fig. 1. Design and optimization of the FACE system. (A) Schematic representation of the FACE system. The engineered bacterial photoreceptor BphS is activated by FRL (~730 nm) to convert GTP into c-di-GMP. Increased cytosolic c-di-GMP production dimerizes the FRL-dependent transactivator p65–VP64–NLS–BldD, enabling it to bind to its chimeric promoter P_FRLx to initiate expression of the FRL-inducible genome transactivator (FGTA4) by assembling MS2, p65, and HSF1 into different protein configurations which are further recruited by the MS2 box of the sgRNA–dCas9 complex to induce transgene expression. (B) Different configurations of the FRL-inducible genome transactivators (FGTAx). (C) Different configurations of the FRL-inducible genome promoters P_FRLx. (D–H) Optimization of the P_FRLx-driven FGTAx expression vector variants for the FACE system. HEK293 cells (6 × 10⁴) were cotransfected with pWS46 (P_hCMV–BphS–2A–YhjH–pA), pGY32 (P_hCMV–FRTA3–pA), pSZ69 (P_U6–sgRNA1 (ASCL1)–pA), and pWS137 (P_FACE–SEAP–pA) and then were illuminated for 6 h daily. SEAP expression in the culture supernatants was assayed at 48 h after the first illumination. (I–K) Optimization of FRL-specific promoter (P_FRLx)-driven FGTA2.4 (MS2–p65–HSF1) for the FACE system. HEK293 cells (6 × 10⁴) were cotransfected with pWS46, pGY32, pSZ83 (P_U6–sgRNA1 (ASCL1)–pA), pSZ84 (P_U6–sgRNA2 (ASCL1)–pA), pSZ69, and pGY102 at a 4:4:2:2:2:1 ratio (wt/wt/wt/wt/wt/wt) and then were illuminated with FRL (1.5 mW/cm², 730 nm) daily. SEAP expression in the culture supernatants was assayed at 48 h after the first illumination. The blue frame in K marks the best-in-class condition chosen for the subsequent experiments. All data are expressed as means ± SEM; n = 3 independent replicate experiments. (L) Activation of the endogenous ASCL1 gene by targeting sgRNAs under FRL illumination with the FACE system. HEK293 cells (6 × 10⁴) were cotransfected with pWS46, pGY32, pSZ69 (P_U6–sgRNA1 (ASCL1)–pA), pSZ84 (P_U6–sgRNA2 (ASCL1)–pA), pSZ69, and pGY102 at a 4:4:2:2:2:1 ratio (wt/wt/wt/wt/wt/wt) and then were illuminated with FRL (1.5 mW/cm², 730 nm) daily. Relative mRNA expression levels compared with that in the dark were quantitated by qRT-PCR and are presented as means ± SEM; n = 3 independent replicate experiments.
could be used to modulate the in vivo activation of endogenous target genes through transepigenetic remodeling. This study focused on endogenous genes involved in muscle mass and regeneration in an attempt to treat several myopathies or muscle loss associated with chronic diseases. We targeted the mouse laminin subunit alpha 1 (Lama1) gene or follistatin (Fst) gene because increased expression of Lama1 could provide new mechanical linkages between the extracellular matrix and the sarcolemma (30), while overexpressed Fst could increase muscle mass (14). The tibialis posterior muscles of the mice were electroplated with a total of 40 μg of the plasmids containing the FACE system or the CPTS 2.0 system with a group of two sgRNAs that specifically target the promoter regions of the Lama1 or Fst genes. This was followed by illumination with FRL or blue light (10 mW/cm²) for 4 h each day (Fig. 4C). The qPCR results showed that the endogenous Lama1 (Fig. 4D) or Fst (Fig. 4E) gene was significantly up-regulated by the FACE system (approximately twofold for Lama1 and approximately fivefold for Fst, compared with mice in the dark). However, CPTS2.0 did not up-regulate Lama1 or Fst gene expression in muscle tissues compared with mice in the dark. Collectively, these data indicate that the FACE system might be used for targeting epigenetic modifications and further confirm that the FACE system is much superior to the previously reported CPTS 2.0 blue light-inducible system, particularly for in vivo applications.

**Optogenetic Control of Neuronal Differentiation of iPSCs with the FACE System.** Despite great potential, the generation of functional neurons from iPSCs for transplantation is limited in clinical applications due to the long duration of culture required for neuronal induction and the low efficiency of neuronal-lineage differentiation. Much ongoing effort has been focused on optimizing culture protocols to enhance neural-lineage differentiation from stem cells (31, 32). The ability to selectively up-regulate gene expression provides another promising strategy to direct somatic or stem cell differentiation for regenerative medicine applications. Previous studies have shown that ectopic expression of neurogenin 2 (NEUROG2) or neurogenic differentiation factor 1 (NEUROD1) is sufficient to induce neuronal differentiation from iPSCs (33–35). Therefore, in this study, we tested whether the FACE system with sgRNAs targeting NEUROG2 could optogenetically initiate functional neuronal differentiation from mouse iPSCs (Fig. 5A). Firstly, we optimized and confirmed that iPSCs transfected with the FACE system without YhjH exhibited brighter EGFP fluorescence intensity under FRL illumination than cells incubated in the dark (SI Appendix, Fig. S5). Next, we generated stable iPSCs lines integrated with the FACE system, which were then transfected with lentiviral vectors containing a mixed pool of two sgRNAs that target NEUROG2. To confirm the efficiency of neuronal-lineage differentiation, the stable iPSC cell lines containing sgRNAs were exposed to FRL and were closely monitored for biochemical and phenotypic changes. qRT-PCR analysis revealed that the NEUROG2 mRNA expression level was significantly up-regulated in the illuminated cells compared with cells in the dark (Fig. 5B). We also observed that the stable iPSCs exhibited typical morphological features of a neuronal cell under FRL illumination (Fig. 5C). In addition, these cells stained positively for the neuronal markers neurofilament 200 and beta III tubulin (Tuj1) after 8 d of FRL illumination (Fig. 5C). Moreover, the expression of the blue light-controlled system (CPTS 2.0) (20) in vivo. Optogenetically engineered cells seeded in hollow fibers were implanted into the dorsum of mice and exposed to FRL or blue light for 2 h (5 mW/cm²) (Fig. 4A). As shown by the data (Fig. 4B), our FACE system demonstrated very efficient endogenous gene ASCL1 up-regulation of about 195-fold compared with mice in the dark. This is mainly due to deep penetration by FRL. In contrast, the CPTS 2.0 blue light-controlled system showed only ninelfold ASCL1 up-regulation. We then further examined whether the FACE system could be used to modulate the in vivo activation of endogenous target genes through transepigenetic remodeling. This study focused on endogenous genes involved in muscle mass and regeneration in an attempt to treat several myopathies or muscle loss associated with chronic diseases. We targeted the mouse laminin subunit alpha 1 (Lama1) gene or follistatin (Fst) gene because increased expression of Lama1 could provide new mechanical linkages between the extracellular matrix and the sarcolemma (30), while overexpressed Fst could increase muscle mass (14). The tibialis posterior muscles of the mice were electroplated with a total of 40 μg of the plasmids containing the FACE system or the CPTS 2.0 system with a group of two sgRNAs that specifically target the promoter regions of the Lama1 or Fst genes. This was followed by illumination with FRL or blue light (10 mW/cm²) for 4 h each day (Fig. 4C). The qPCR results showed that the endogenous Lama1 (Fig. 4D) or Fst (Fig. 4E) gene was significantly up-regulated by the FACE system (approximately twofold for Lama1 and approximately fivefold for Fst, compared with mice in the dark). However, CPTS2.0 did not up-regulate Lama1 or Fst gene expression in muscle tissues compared with mice in the dark. Collectively, these data indicate that the FACE system might be used for targeting epigenetic modifications and further confirm that the FACE system is much superior to the previously reported CPTS 2.0 blue light-inducible system, particularly for in vivo applications.
specific neural-lineage markers including Sox1, Pax6, N-cadherin, TuJ1, and Nestin was significantly up-regulated in the differentiated neurons compared with cells in the dark (Fig. S5D). Finally, to further verify the functionality of the FRL-induced neuronal cells, calcium ion imaging was performed to monitor neuronal activity in the generated neurons in vitro. The results showed that the differentiated neuronal cells displayed a significant change in the calcium ion concentration within the cytosol upon treatment with 50 mM KCl (Fig. 5E). In conclusion, we can easily obtain functional neurons from iPSCs by FRL illumination with the FACE system.

Discussion

An ideal optogenetic CRISPR-dCas9 system would be orthogonal and provide fine-tunable and robust transcriptional control of selected genes in response to light with deep tissue penetration, low brightness, short illumination time, negligible phototoxicity, and excellent biocompatibility. In this study, we report the engineering of a light-controllable transcriptional activation system. Our FACE system is easily programmable, which provides a straightforward and robust method to regulate the simultaneous activation of user-defined endogenous genes with precise spatiotemporal control. Our system exhibits high induction efficiency upon illumination in various human cell lines with reasonably low background activity in the dark. Therefore this may be a suitable option if gene regulation with high temporal resolution is required to regulate endogenous gene expression precisely.

It should be possible to apply our FACE system to control gene transcriptional regulation in vivo, as light of wavelengths >600 nm is able to propagate further into tissues more efficiently than blue light (37). Compared with the blue light-controlled endogenous gene activation system (20), our FACE system can robustly trigger endogenous gene activation in mice, indicating many potential applications in vivo. Therefore, the FACE system developed in this study can pave the way for optogenetics-based translational research, and we anticipate that this system holds great promise for safe medical applications in humans.

**Fig. 3.** FRL-inducible, RNA-guided activation of endogenous genes by the FACE system. (A) Illumination-dependent FACE-induced endogenous ASCL1 expression. HEK293 cells were cotransfected with pWS46, pGY32, pGY102, pSZ69, pSZ83, and pSZ84 at a 4:4:1:2:2:2 (wt/wt/wt/wt/wt/wt) ratio, followed by illumination with FRL at different light intensities (0–2 mW/cm²) for 6 h daily. (B) Exposure time-dependent FACE-induced endogenous ASCL1 expression. HEK293 cells were cotransfected as described in A and were illuminated with FRL (1.5 mW/cm²) for different time periods every day. (C) FACE-induced endogenous ASCL1 expression in different mammalian cell lines. Different mammalian cell lines were cotransfected as described in A and were exposed to FRL (1.5 mW/cm²; 730 nm) for 6 h every 24 h. (D) Reversibility of the FACE-induced endogenous ASCL1 expression. HEK293 cells were cotransfected as described in A, were illuminated with FRL (1.5 mW/cm²) for 20 min, and then were kept in the dark. After 48 h of incubation, the cells were exposed to FRL for another 20 min. The endogenous ASCL1 levels were quantified by qRT-PCR every 6 h. (E) Multiplexed endogenous gene activation in HEK293 cells containing the FACE system and the indicated sgRNAs. HEK293 cells were cotransfected with pWS46, pGY32, pGY102, and pSZ69 and with the corresponding sgRNAs: pSZ83 and pSZ84 or pSZ292 [pU6-sgRNA1 (IL1RN–)–pA] and pSZ293 [pU6-sgRNA2 (IL1RN–)–pA] or pSZ2105 [pU6-sgRNA1 (RHOXF2–)–pA] and pSZ2106 [pU6-sgRNA2 (RHOXF2–)–pA] or pSZ107 [pU6-sgRNA1 (TTN–)–pA] and pSZ108 [pU6-sgRNA2 (TTN–)–pA] or all eight mixed sgRNAs, followed by exposure to FRL (1.5 mW/cm²; 730 nm) for 6 h every day. The data are expressed as relative mRNA expression levels quantified by qRT-PCR at 48 h after the first illumination. All data are presented as the mean ± SD (n = 3 independent replicate platings of each culture).
...for a broad array of in vivo biomedical research applications in the future.

Conditional dCas9-based methodologies have broad applications in diverse biomedical fields (38, 39). For instance, inducible systems can be used to investigate embryonic development or stem cell differentiation and dissect the dynamic regulatory network orchestrating vertebrate development. Our FACE system also provides a robust method for generating functional neural cells from iPSCs under FRL illumination through the up-regulation of a single neural transcription factor. We anticipate that our FACE system with characteristics of reversible, tunable, spatiotemporal control of endogenous gene expression could expand the optogenetic toolkit for precise mammalian genome engineering in many areas of basic and translational research that require precise spatiotemporal control of cellular behavior, in turn boosting the clinical progress of precision optogenetics-based therapy.

Materials and Methods

Construction of Plasmids. Comprehensive design and construction details for all expression vectors are provided in SI Appendix, Table S1. Some plasmids were cloned by Gibson assembly according to the manufacturer’s instructions [Seamless Assembly Cloning Kit; catalog no. BACR(C) 20144001; Obio Technology, Inc.]. All genetic components have been validated by sequencing (Geneviz, Inc.).

Cell Culture and Transfection. HEK293 cells (CRL-11268; ATCC), HEK293-derived HEK293A cells containing a stably integrated copy of the E1 gene (R70507; Thermo Fisher), HeLa (human cervical adenocarcinoma) cells (CCL-2; ATCC), telomerase-immortalized human mesenchymal stem cells (hMSC-TERT) (catalog. no. 31600-083; Gibco) supplemented with 10% (vol/vol) FBS (catalog no. 04-001-1C; Biological Industries) and 1% (vol/vol) penicillin/streptomycin solution (catalog. no. L0022-100; Biowest). All cell types were cultured at 37 °C in a humidified atmosphere containing 5% CO2 and were regularly tested for the absence of Mycoplasma and bacterial contamination. All cell lines were transfected with an optimized polyethyleneimine (PEI)-based protocol (42). Briefly, 6 × 104 cells per well were plated in a 24-well plate at 18 h before transfection and were subsequently incubated for 6 h with 50 μL of a 3:1 PEI DNA mixture (wt/vt) (PEI, molecular weight 40,000, stock solution 1 mg/mL in ddH2O; catalog. no. 24765; Polysciences) containing 0.375 μg of plasmid DNA (total plasmid amount for FACE). Cell titters and viability were quantified with a Countess II automated cell counter (Life Technologies).

FRL-Controlled Gene Activation in Mammalian Cells. For exogenous gene-activation experiments, 6 × 104 cells were plated in a 24-well plate and were cultured for 18 h. The cells were transfected with 50 μL of a 3:1 PEI-DNA mixture (wt/vt) containing 0.375 μg of plasmid DNA (total plasmids amount for FACE). For endogenous gene-activation experiments, HEK293 cells were cotransfected with 100 ng pWS46 (P_U6–hCMV–dCas9–HSF1–pA/p65–MS2–NES–MS2–pA–P_U6–dCas9–hCMV–pA; FRTA3: MS2–hCMV–pA; 3:1 PEI mixture) into a 10-cm dish, cultured for 18 h, and then transfection performed with 50 μg of plasmid DNA (total plasmids amount for FACE). At 24 h after transfection, cells were harvested for different time periods (0–6 h) or with different light intensities (0–5 mW/cm2) using a custom-designed 4 × 6 far-red light LED array (730 nm; Epistar) (22). The light intensity was measured at a wavelength of 730 nm using an optical power meter (Q2030; Advantest) according to the manufacturer’s operating specifications. SEAP expression levels in the culture medium were quantified at 48 h after transfection (SI Appendix, Fig. S6). For endogenous gene-activation experiments, HEK293 cells were cotransfected with 100 ng pWS46 (P_U6–hCMV–dCas9–HSF1–pA; P_U6–hCMV–pA; P_U6–dCas9–pA; P_U6–hCMV–pA; FRTA3: MS2–hCMV–pA/p65–MS2–NES–pA) or 100 ng pWS46 (P_U6–hCMV–pA/p65–MS2–NES–pA) and 50 ng of the corresponding sgRNAs (SI Appendix, Table S3). The transfected cells were cultured for 24 h after transfection. The culture plates were then placed below a custom-designed 4 × 6 LED array. Each plate (maintained at 37 °C and 5% CO2) was illuminated (1.5 mW/cm2; 730 nm) for 6 h, once daily, for 2 d (SI Appendix, Fig. S6). The cells were harvested, and total RNA was extracted for qPCR analysis at 48 h after the first illumination.

Spatial Control of FRL-Dependent Gene Activation in Mammalian Cells. HEK293 cells (3 × 106) were plated into a 10-cm dish, cultured for 18 h, and transfected with 1,500 μL of a 3:1 PEI-DNA mixture (wt/vt) containing 11.25 μg of plasmid DNA (total plasmid amount for FACE). At 24 h after transfection, the cells were illuminated for 6 h each day by FRL (1.5 mW/cm2; 730 nm) with a photomask with the pattern “ECNU” made from aluminum foil. The patterned photomask was placed between the upward-facing LED array and the bottom of the 10-cm culture dish (Fig. 2E). Fluorescence images were acquired at 48 h after illumination using ChemiScope 4300 Pro imaging equipment (Clinx).
Fig. 5. FRL-stimulated iPSCs differentiate into functional neurons with the FACE system. (A) Schematic representation of FRL-induced neuronal differentiation from iPSCs with the FACE system. (B) FRL-induced NEUROG2 up-regulation in iPSCs with the FACE system and two sgRNAs targeting NEUROG2 after 1, 4, and 8 d of FRL illumination for 6 h every day or incubation in the dark. Relative mRNA expression levels compared with those in the dark were quantified by qRT-PCR and are presented as means ± SD; n = 3 independent replicate experiments; ***P < 0.001. (C) Immunofluorescence images of iPSCs showing the positive expression of neuronal markers neurofilament (magenta) and Tuj1 (green) after 8 d of FRL illumination. Cell nuclei were counterstained with DAPI (blue). Stable iPSC lines containing the FACE system were transduced with lentiviral preparations containing two sgRNAs targeting NEUROG2. The images are representative of at least three replicate experiments. (Scale bars, 250 μm.) (D) qRT-PCR analysis of mRNA expression levels of neuronal marker genes (Sox1, Pax6, N-cadherin, Tuj1, and Nestin) by iPSC lines after 8 d of FRL illumination. All data are presented as the mean ± SD; n = 3 independent replicate experiments. ***P < 0.001. (E) Calcium ion imaging analysis of FRL-induced neurons by Fluo-4 AM. The iPSC-derived functional neurons treated with 50 mM KCl exhibited changes in Ca²⁺ transients upon depolarization. Ca²⁺ response is expressed as relative change in the fluorescence emission intensity above the normalized baseline (F/F₀). The plot is representative of three independent replicate experiments.
SEAP Assay. The production of human placental SEAP in cell-culture medium was assayed using a p-nitrophenyl phosphoric acid-based light-absorbance time-course method as described previously (22, 43). Briefly, 120 μL of substrate solution [100 μL of 2× SEAP assay buffer including 20 mM homomannosine, 1 mM MgCl₂, 21% (wt/vol) diethanolamine, pH 9.8] and 20 μL of substrate solution containing 120 mM p-nitrophenyl phosphoric acid was added to 80 μL of heat-deactivated (65 °C, 30 min) cell-culture supernatant, and the light absorbance was measured at 405 nm (37 °C) for 30 min using a Synergy H1 hybrid multimode microplate reader (BioTek Instruments, Inc.) using Gen5 software (version: 2.04).

Cell Viability Assay. HEK293 cells (1 × 10⁵) were seeded into each well of a 96-well plate. At 24 h after culture, cells were exposed to FRL (1.5 mM, 730 nm) or blue light (1.5 mM, 465 nm) for 48 h, and cell viability was evaluated with the Cell Counting Kit-8 (Beyotime) according to the manufacturer’s protocol. After treatment with CCK8 at 37 °C for 2 h, the absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, Inc.).

qRT-PCR Analysis. Cells were harvested for total RNA isolation using an RNeasy Plus kit (catalog no. 9089; Takara) according to the manufacturer’s instructions. A total of 1 μg RNA was reverse transcribed into cDNA using a PrimeScript RT Reagent Kit with the genomic DNA Eraser (catalog no. RR047; Takara) according to the manufacturer’s protocol. qPCR analysis was performed according to the QuantiStudio 3 real-time PCR instrument (Thermo Fisher Scientific Inc.) using the SYBR Premix Ex Taq (catalog no. RR420; Takara) for detecting each target gene. Conditions for PCR amplifications were as follows: 95 °C for 10 min, 40 thermal cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. The qRT-PCR primers used in this study are listed in SI Appendix, Table S2. All samples were normalized to housekeeping GAPDH values, and the results are expressed as the relative mRNA level normalized to that in the dark using the standard ΔΔCT method.

Hollow Fiber Implants. Optogenetically engineered HEK293 cells (1 × 10⁶) containing the FACE system or the blue light-inducible dCas9 transcription system CPTS 2.0 were seeded into a 2.5-mm semipermeable KrosFlo hollow fiber (Spectrum Laboratories Inc.), and both ends of the hollow fiber were system CPTS 2.0 were seeded into a 2.5-cm semipermeable KrosFlo hollow fiber. The experiments involving animals were approved by the Experimental Animal Care guidelines. The protocol (protocol ID: 20171012) was assayed using a p-nitrophenyl phosphate-based light-absorbance time-course method as described previously (22, 43). Briefly, 120 μL of substrate solution (100 μL of substrate solution, maintained on irradiated mouse embryonic fibroblast feeder cells in 0.1% (wt/vol) gelatin-coated dishes in Glasgow Minimum Essential Medium (catalog no. 11710-035; Gibco), supplemented with 15% (vol/vol) FBS (catalog no. 16000-044; Gibco), 1% (vol/vol) nonessential amino acids (catalog no. 11140-050; Gibco), 1 × 10⁻³ M GlutaMAX (catalog no. 35050-061; Gibco), and 0.1 × 10⁻⁵ M β-mercaptoethanol (catalog no. M3148; Sigma), and 1.0 μM recombinant mouse leukemia inhibitory factor (LIF; catalog no. ES6110; Millipore). The medium was changed daily. To generate stable iPSCs incorporating the FACE construct, ~5 × 10⁶ cells were nucleofected with 100 ng of pW5287 [LTR-NeoR-p65-VP64-NLS-BBD-pA-NeoR-BphS-pA-NeoR-PuroR-PA-ITR], 100 ng of pW5289 [LTR-NeoR-TG-FTA4-pA-NeoR-ZeoR-PA-ITR; pFLRC-pA-(w(hi)g)], pW5294 [FGTA4], MS2-P65-HSF1-ITR, and 20 ng of the Sleeping Beauty transposase expression vector pCMV-T7–SV8000 (pCMV-T7–SV8000–pa) using the P3 Primary Cell 4D-Nucleofector X Kit S (Lonza) and the CG4-104 program. Following electroporation, these cells were seeded into 24-well gelatin-coated cell-culture plates in the presence of 1 μg/mL puromycin and 100 μg/mL zeocin to select for a mixed population.

IPSC Transduction and Optogenetic Neural Induction. The stable transgenic iPSCs line with the FACE system was transduced with lentivirus containing dCas9 [pXS187 (LTR–P2Ac–dCas9–p2A–Blast–LTR)] and two sgRNAs [pW574 (LTR–P2Ac–sgRNA1 (NEURO2G)–LTR) and pW750 (LTR–P2Ac–sgRNA2 (NEURO2G)–LTR)] targeting NEURO2G (SI Appendix, Table S3) at 1 d after seeding into 24-well gelatin-coated cell-culture plates in the presence of 1 μg/mL puromycin and 100 μg/mL zeocin. Transduced cells were incubated at 37 °C in 5% CO₂ under FRL light illumination for 6 h each day or in the dark. Fresh growth medium without LIF was changed daily for 8 d. In the last 4 d, 5 × 10⁻⁷ M of all-trans retinoic acid (catalog no. R2625; Sigma) was added to the medium. The cells were then analyzed by immunofluorescence staining and qRT-PCR.

Immunofluorescence Staining. Cells were fixed with 4% (v/v) paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 15 min, and then blocked with 10% (vol/vol) goat serum in PBS for 30 min at room temperature. Subsequently, the cells were incubated for 1 h with the primary antibodies anti-beta III tubulin (catalog no. MMS-435P; BioLegend) or anti-neurofilament 200 (catalog no. N4142; Sigma), both at 1:500 dilution. After incubation with the primary antibodies, cells were washed three times with PBS (5 min each time) and then were incubated with the secondary antibodies rabbit anti-mouse Alexa Fluor 488 or goat anti-rabbit Cy3 (1:200; Jackson ImmunoResearch) for 1 h. After three 5-min washings with PBS, the cell nuclei were counterstained with DAPI (0.25 μg/mL; Molecular Probes) for 10 min and were imaged under a 20× objective on an inverted fluorescence microscope (Leica DMIB).

Calcium Ion Imaging. After differentiation, the putative neuronal cells were washed three times with 1x HBSS (catalog no.14175095; Gibco) and then were stained with 3 μM of the calcium indicator Fluo-4 AM loading solution (catalog no. F14201; Gibco) for 60 min at 37 °C. Excess dye solution was then removed from the dish, and the cells were finally washed three times with 1x HBSS. The cells were exposed to a 50-nM KC1 solution before imaging with a Leica DMIB Microscope. Time-lapse imaging was performed at an excitation wavelength of 488 nm for 5 min. The data were analyzed using the Leica LAS AF software.

Statistical Analysis. All data are expressed as the mean ± SD, with sample sizes indicated in the figure legends. All qualitative images presented are representative of at least two independent duplicate experiments. Statistical significance was analyzed by the Student’s t test. Prism 5 software (version: 5.01; GraphPad Software Inc.) was used for statistical analysis. No sample exclusion was carried out, nor was randomization or blinding utilized in this study.

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