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An Engineered Optogenetic Switch for Spatiotemporal Control of Gene Expression, Cell Differentiation, and Tissue Morphogenesis

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Abstract

The precise spatial and temporal control of gene expression, cell differentiation, and tissue morphogenesis has widespread application in regenerative medicine and the study of tissue development. In this work, we applied optogenetics to control cell differentiation and new tissue formation. Specifically, we engineered an optogenetic “on” switch that provides permanent transgene expression following a transient dose of blue light illumination. To demonstrate its utility in controlling cell differentiation and reprogramming, we incorporated an engineered form of the master myogenic factor MyoD into this system in multipotent cells. Illumination of cells with blue light activated myogenic differentiation, including upregulation of myogenic markers and fusion into multinucleated myotubes. Cell differentiation was spatially patterned by illumination of cell cultures through a photomask. To demonstrate the application of the system to controlling *in vivo* tissue development, the light inducible switch was used to control the expression of VEGF and angiopoietin-1, which induced angiogenic sprouting in a mouse dorsal window chamber model. Live intravital microscopy showed illumination-dependent increases in blood-perfused microvasculature. This optogenetic switch is broadly useful for applications in which sustained and patterned gene expression is desired following transient induction, including tissue engineering, gene therapy, synthetic biology, and fundamental studies of morphogenesis.

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Conflict of Interest

The authors declare no conflicts of interest.

Supporting Information

Supplementary Methods describing plasmid construction, cell culture, transductions, transfections, and gene expression analysis.

Figure S1 showing light-dependent expression of MyoD using the light-activated genetic “on” switch (Flox) vector.

Figure S2 showing inefficient myogenesis in C3H10T $\frac{1}{2}$ cells using Flox_M.

Figure S3 showing VEGF and ANG1 expression in HEK 293T cells containing the Flox_{VPA} vector.

All novel DNA sequences.

Keywords

optogenetics; recombination; light-inducible; myogenesis; angiogenesis; cell transplantation

Introduction

Gene expression in multicellular systems is tightly controlled both spatially and temporally. Many synthetic gene regulation systems have been developed to simulate this control for diverse applications in science, medicine, and biotechnology¹. Optogenetic systems are unique in their ability to provide both spatial and temporal control of gene expression². Most of these systems use light-inducible, noncovalent protein-protein interactions that are reversible and allow for dynamic control of gene expression, such that gene activation or repression is always dependent on illumination^{3–11}. Other systems use irreversible, covalent protein modifications, but the effect only lasts for the lifetime of the modified proteins^{12, 13}. However, many applications require long-term changes in gene expression to achieve desired outcomes, and the necessity to maintain the exogenous illumination stimulus can be prohibitive. In these cases, it would be useful to initiate the induction of gene expression at any time with transient illumination in a defined spatial pattern, and then maintain gene expression in this pattern indefinitely in the absence of stimulus.

In particular, applications such as tissue engineering require spatial control of gene activation in order to recapitulate the heterogeneous complexity and architecture of the tissues they are intended to model or replace. Consequently, a light-inducible gene expression system that behaves as a permanent “on” switch upon illumination would serve as a useful optogenetic tool. In addition to tissue engineering, this approach could be useful for applications in gene therapy, synthetic biology, and the study of tissue development and morphogenesis.

To develop this tool we used a light-activated genetic “on” switch that works in concert with a previously described light-inducible split Cre recombinase system¹⁴. This system takes advantage of the light-inducible plant heterodimerizers CRY2 and CIB1¹⁵ and the inducible reassembly of a split Cre recombinase enzyme¹⁶. CRY2 is fused to an N-terminal fragment of Cre (CRY2-Cre^N), and either the first 170 N-terminal amino acids of CIB1 (CIB1^N) or the full-length CIB1 (CIB1^{FL}) is fused the C-terminal fragment of Cre (CIB1^N-Cre^C and CIB1^{FL}-Cre^C, respectively) (Fig. 1A,B). In the absence of blue light, CRY2 and CIB1 are not bound, and the split Cre recombinase system is inactive. Illumination with blue light causes heterodimerization of CRY2 and CIB1, which reconstitutes the full-length Cre recombinase and restores its activity. Consequently, this system enables light-inducible Cre recombinase-mediated excision of DNA segments flanked by LoxP sequences¹⁴.

In order to create an optogenetic “on” switch, we interrupted a lentiviral gene expression cassette with the coding sequences for green fluorescent protein (GFP) and the puromycin resistance gene (Puro^r), separated by an internal ribosomal entry site (IRES) and flanked by LoxP sites (Fig. 1C). Thus engineered cells are initially GFP-positive and puromycin-resistant. Following activation of the “on” switch and light-mediated recombination, the GFP-IRES-Puro^r cassette is removed and the downstream gene is upregulated by the

upstream promoter. Because the target for recombination is integrated into the genome by lentiviral transduction, an initial dose of illumination causes the switch to permanently remain on as the cells continue to divide. This contrasts with methods that use transient transfection of expression plasmids, which degrade and dilute as the cells proliferate. The recombination system also differs from reversible light-inducible transcriptional activation systems^{7, 9, 14, 17–22} in that it does not require sustained illumination to achieve lasting transgene expression.

A primary motivation for developing this genetic “on” switch is to achieve control of biological processes that require modulation of gene expression for days or weeks, such as directing cell differentiation, reprogramming cell fate specification, and inducing complex tissue development. As a model system for directing cell differentiation, we focused on differentiation to skeletal myocytes by the master transcription factor MyoD. MyoD overexpression has been used to reprogram cell fate^{23–28}, generate myogenic cells for cell therapy^{29–32}, and engineer models of skeletal muscle³³. This is also a useful model system given the well-defined changes to gene expression and cell morphology associated with MyoD-induced skeletal myocyte differentiation.

Similarly, angiogenesis is an ideal model system for studying gene regulation and tissue morphogenesis *in vivo* given the well-studied signaling pathways involved and their relevance to tissue regeneration, wound healing, and cancer. Sustained expression of Vascular Endothelial Growth Factor (VEGF), Angiopoietin-1 (ANG1), and other angiogenic factors is required for new blood vessels to form and mature³⁴. Forced expression of these factors is sufficient to induce new blood vessel formation^{34–39}. Moreover, insufficient levels of angiogenic factors during vessel development can cause vascular endothelial cell apoptosis and result in retraction and degradation of the newly formed vessels^{40, 41}.

We showed that the Flox system behaves as a true “on” switch with low levels of transgene expression prior to light exposure and robust transgene expression upon illumination with blue light. We achieved light-inducible myogenic differentiation *in vitro* as well as light-inducible angiogenesis *in vivo* using a mouse dorsal window chamber model.

Results

Design of the Optogenetic Switch

The light-activated genetic “on” switch is a lentiviral vector designed to switch from an “off” to “on” state upon Cre-mediated recombination (Fig. 1C). This vector contains a constitutive human Ubiquitin C (hUbC) promoter upstream of an eGFP-IRES-Puro^r cassette that is flanked by LoxP sites (Flox Vector). Any transgene of interest that is desired to be turned “on” by the light-inducible Cre recombinase system can be cloned downstream of the second LoxP site of the cassette. Importantly, the start codon of the downstream transgene is located upstream of the first LoxP site, approximately 2 kb upstream of the rest of the coding sequence. Thus, in the absence of Cre recombinase, the entire ATG-LoxP-eGFP-IRES-Puro^r-LoxP-transgene mRNA is expected to be transcribed. However, only the eGFP and Puro^r mRNAs would be translated into proteins. Following Cre-mediated recombination, the eGFP-IRES-Puro^r cassette is excised and the ATG start codon comes into

position at the beginning of the transgene, resulting in translation of the corresponding protein with a predicted 17 additional amino acids at the N-terminus that encode the residual LoxP site and cloning sequences.

Characterization of the Optogenetic Switch

The optogenetic switch can be used to activate any desired transgene by illuminating cells that contain the Flox vector, CRY2-Cre^N, and CIB1-Cre^C. To characterize this system, we engineered a Flox vector containing a mouse MyoD transgene (Flox_M) to induce *in vitro* myogenic differentiation. We first created a polyclonal Flox_M cell line by transducing human HEK293T cells with a lentivirus containing the Flox_M vector. Transfection of this Flox_M-modified HEK293T cell line with plasmids encoding CRY2-Cre^N and either CIB1^{FL}-Cre^C or CIB1^N-Cre^C led to blue light-dependent expression of MyoD (Fig. S1). When incubated in the dark, cells that received CIB1^N-Cre^C demonstrated lower background levels of MyoD expression compared to cells that received CIB1^{FL}-Cre^C. However, cells transfected with CIB1^{FL}-Cre^C demonstrated much greater activation levels of MyoD when illuminated compared to cells that received CIB1^N-Cre^C. These results suggest that the fusion of full-length CIB1 has greater overall activity, consistent with previous reports¹⁴. Importantly, cells that only received the Flox_M vector without any Cre fusions showed no evidence of MyoD protein expression, suggesting that the switch has negligible “leakiness” of transgene expression in the absence of Cre activity. Cells containing Flox_M expressed high levels of MyoD when transfected with a plasmid that constitutively expresses the intact, full-length Cre recombinase (CMV-Cre), and these levels were similar whether the cells were incubated in the dark or illuminated with blue light. Lower levels of light-inducible MyoD expression than achieved with CMV-Cre indicate a lower level of DNA recombination.

Light-Inducible Myogenic Differentiation

We next tested the ability of the Flox_M system to induce myogenic differentiation of multipotent C3H10T½ mesenchymal cells into multinucleated, fused myotubes. Initial experiments suggested that this system is capable of activating the myogenic differentiation pathway in response to blue light as determined by qRT-PCR for the myogenic marker myogenin (Fig. S2). However, activation was weak and highly variable, and we were unable to detect cellular fusion into myotubes or positive immunofluorescence staining of any downstream myogenic markers typical of myogenic differentiation. We therefore modified the Flox vector to express the enhanced synthetic MyoD fusion protein^{VP64MyoD⁴²} (Flox_{VM}) in response to blue light. VP64MyoD is more potent than MyoD in stimulating conversion of human dermal fibroblasts and adult stem cells to a skeletal muscle phenotype. In addition to engineering the new Flox_{VM} vector, we also cloned CRY2-Cre^N and CIB1^{FL}-Cre^C into a lentiviral vector that drives transgene expression from a human Ubiquitin C (hUbC) promoter (LV_{hUbC}) to achieve robust and sustained expression of these components of the optogenetic switch. Lastly, because CIB1^{FL}-Cre^C yielded higher recombination of the Flox vector (Fig. S1, S2), we used this fusion protein along with CRY2-Cre^N for the remainder of these experiments.

C3H10T $\frac{1}{2}$ cells were transduced with Flox_{VM} and LV_{hUbc}-CIB1^{FL}-Cre^C, expanded, and then transfected with LV_{hUbc}-CRY2-Cre^N. We stably expressed only CIB1^{FL}-Cre^C so that the cells could be propagated under normal light conditions until they were transfected with CRY2-Cre^N. The day after transfection, cells were either illuminated with blue light for two days (1 sec pulses, 0.067 Hz) and then incubated in the dark for five more days or were incubated in the dark for the entire experiment. Illuminated C3H10T $\frac{1}{2}$ cells that were transduced with Flox_{VM} and LV_{hUbc}-CIB1^{FL}-Cre^C and transfected with LV_{hUbc}-CRY2-Cre^N showed significant activation of the downstream myogenic markers myogenin, troponin T, desmin, and myosin heavy chain II (MHCII) compared to the same cells that were incubated in the dark ($p < 0.01$) (Fig. 2A). Importantly, the dark-incubated cells maintained mRNA expression levels of all four genes that were statistically equivalent to cells that were transduced with Flox_{VM} and LV_{hUbc}-CIB1^{FL}-Cre^C and transfected with an empty plasmid as a negative control ($p > 0.74$), demonstrating low “leakiness” of the system when incubated in the dark. Cells transduced with Flox_{VM} and LV_{hUbc}-CIB1^{FL}-Cre^C and transfected with CMV-Cre expressed significantly higher levels of myogenin, MHCII, and troponin T compared to illuminated cells that were transduced with Flox_{VM} and LV_{hUbc}-CIB1^{FL}-Cre^C and transfected with CRY2-Cre^N ($p = 0.02$, $p < 0.0001$, and $p = 0.03$, respectively), indicating lower levels of recombination by the optogenetic switch compared to constitutive Cre activity. Lastly, illuminated cells had significantly higher levels of the recombined form of Flox_{VM} when compared to the same cells incubated in the dark (Fig. 2B).

Illuminated cultures transduced with Flox_{VM} and LV_{hUbc}-CIB1^{FL}-Cre^C and transfected with LV_{hUbc}-CRY2-Cre^N or CMV-Cre contained many myogenin-positive nuclei as visualized by immunofluorescence staining (Fig. 2C). Some myogenin-positive cells also expressed MHCII and fused into multinucleated cells that resembled skeletal myocytes. Importantly, there was a low level of background myogenin and MHCII staining in dark-incubated cells that were transduced with Flox_{VM} and LV_{hUbc}-CIB1^{FL}-Cre^C and transfected with LV_{hUbc}-CRY2-Cre^N or an empty plasmid.

Patterned Myogenic Differentiation

A unique property of optogenetic control is the ability to spatially pattern gene expression. To achieve spatially patterned cell differentiation, C3H10T $\frac{1}{2}$ cells transduced with Flox_{VM} and LV_{hUbc}-CIB1^{FL}-Cre^C and transfected with LV_{hUbc}-CRY2-Cre^N were illuminated through a photomask for two days to allow for recombination and then incubated in the dark for seven days. Immunofluorescence staining showed a higher density of myogenin- and MHCII-positive cells within the illuminated region compared to regions that were shielded from light exposure (Fig. 2C,D; $p < 0.001$). Staining of all cell nuclei with DAPI showed that there was no statistical difference in cell density within each region.

Light-Inducible VEGF₁₆₅ and ANG1 Flox Vector

To test the optogenetic switch for controlling gene expression and tissue morphogenesis *in vivo*, we engineered Flox_{VP_A}, which expresses human VEGF isoform 165 (VEGF) and human ANG1 from a VEGF-P2A-ANG1 cassette in response to Cre-mediated recombination. P2A is a short peptide sequence that facilitates ribosomal skipping⁴³.

Placement of a P2A coding sequence between the VEGF and ANG1 transgenes produces two separate protein products from a single mRNA transcript: (1) VEGF with the P2A peptide added to its C-terminus, and (2) ANG1 with a single proline added to its N-terminus. We chose to express VEGF along with ANG1 after a pilot experiment in which constitutive expression of VEGF alone within the mouse dorsal window chamber model led to leaky blood vessel formation and fluid accumulation, as observed previously^{34, 35, 40, 41}. Co-expression of VEGF and ANG1 improves therapeutic effects and creates more functional blood vessels^{34, 41}.

Characterization of the Stable Flox_{VP}A Polyclonal Cell Line

We transduced HEK293T cells with Flox_{VP}A to create a polyclonal cell line that could be transfected with CRY2-Cre^N and either CIB1^N-Cre^C or CIB1^{FL}-Cre^C and serve as an implantable source of light-inducible VEGF and ANG1 release into the surrounding environment. We hypothesized that release of VEGF and ANG1 from implanted Flox_{VP}A-modified HEK293T cells *in vivo* would promote host cell migration and vascular formation. Because the Flox_{VP}A lentiviral vector is integrated into the cell genome, activation of the switch provides VEGF and ANG1 production that is sustained upon removal of blue-light illumination. We introduced the light-inducible Cre constructs via transient transfection instead of stable lentiviral transduction to (1) facilitate propagation and preparation of the cells under ambient light without inducing recombination, (2) avoid accumulation of background recombination events in the dark as the cells are cultured and expanded, (3) limit unnecessary stable expression of CRY2-Cre^N and either CIB1^{FL}-Cre^C or CIB1^N-Cre^C, and (4) take advantage of high transfection efficiencies of HEK293T cells.

To test if the Flox_{VP}A vector would behave as a permanent “on” switch, the polyclonal Flox_{VP}A-modified HEK293T cell line was transfected with CRY2-Cre^N and either CIB1^N-Cre^C or CIB1^{FL}-Cre^C, illuminated with pulsing blue light for three days, and then incubated in the dark for 9 more days (Fig. 3A). Identically modified cells were incubated in the dark for the entire experiment for comparison. Unmodified and Flox_{VP}A-modified HEK293T cells transfected with empty plasmid served as negative controls. Flox_{VP}A-modified cells transfected with CMV-Cre served as a positive control. The cells transfected with CRY2-Cre^N and CIB1^N-Cre^C and incubated in the dark produced VEGF and ANG1 levels equivalent to unmodified HEK293T cells transfected only with an empty plasmid. However, when illuminated, these cells containing all three vectors produced ~2.5- and ~7-fold higher levels of VEGF and ANG1, respectively, as determined by ELISA that measured the amount of the proteins in cell supernatant.

Flox_{VP}A-modified cells transfected with CRY2-Cre^N and CIB1^{FL}-Cre^C and incubated in the dark expressed ~30% higher VEGF levels and up to 9-fold higher ANG1 levels than unmodified cells transfected only with an empty plasmid. When illuminated, these cells increased VEGF and ANG1 production ~2.8- and ~7.7- fold, respectively, above identically modified cells that were incubated in the dark. Due to the elevated background expression associated with using CIB1^{FL}-Cre^C, we used CIB1^N-Cre^C in all future VEGF and ANG1 experiments.

It was unexpected that cells containing only the Flox_{VP}A vector exhibited elevated VEGF levels relative to the Flox_{VP}A-modified cells that were transfected with both CRY2-Cre^N and CIB1^N-Cre^C and incubated in the dark. To explore this, we transfected HEK293T cells with Flox_{VP}A alone, Flox_{VP}A and CRY2-Cre^N, Flox_{VP}A and CIB1^N-Cre^C, or Flox_{VP}A with both CRY2-Cre^N and CIB1^N-Cre^C (Fig. 3B, S3). Interestingly, after three days, expression of only one of the light-inducible Cre fusions in the illuminated cells or expression of both light-inducible Cre fusions in dark-incubated cells resulted in the same low background levels of VEGF and ANG1 expression as unmodified HEK 293T cells (Figure S3). Compared to these conditions, cells modified with only the Flox_{VP}A vector resulted in slightly elevated VEGF and ANG1 levels ($p < 0.0001$). As both the N- and C- termini of Cre recombinase contribute to DNA-binding^{44, 45}, this suggests that binding of either fragment at the LoxP site may be responsible for suppressing background VEGF and ANG1 expression. Nevertheless, cells that were transfected with all three vectors and illuminated with blue light produced between 2.5- and 5.5-fold more VEGF protein than cells treated with these other conditions ($p < 0.0001$). These cells also produced 3.5- and 430-fold more ANG1 protein than these other conditions. To confirm that recombination at the target site was in fact occurring in response to light, qRT-PCR was used to detect the recombined Flox_{VP}A transcript. The level of recombined mRNA was elevated in response to illumination at both two and seven days (Fig. 3B).

Light-Inducible Angiogenesis in vivo

We tested light-inducible angiogenesis *in vivo* using a nude mouse dorsal window chamber model⁴⁶. The Flox_{VP}A-modified HEK293T cells were first transfected with CRY2-Cre^N and CIB1^N-Cre^C. The next day, cells were suspended in collagen/fibrin hydrogel constructs within a 1×1 cm frame and then implanted within a window chamber the following day. Mice were split into “light” and “dark” groups (n=4 and n=3 mice, respectively). The “light” group was placed in a cage that was illuminated with pulsing blue light (1 sec pulses, 0.067 Hz pulse frequency) for 8 hours the day of window chamber and hydrogel construct implantation and then 12 hours on each of the following three days. The window chambers of the “dark” group mice were covered by a thin opaque foam sheet. The mice were otherwise exposed to typical ambient light. As positive (n=4 mice) and negative (n=3 mice) controls, constructs were implanted containing the Flox_{VP}A cell line transfected with CMV-Cre or CRY2-Cre^N, respectively.

Live intravital microscopy was performed seven days after implantation (Fig. 4). Images were analyzed for total hemoglobin concentration⁴⁶ and then converted to a binary image (Fig. 4A). Images of the lowest and highest microvessel density are shown for each treatment group. Binary images were used to quantify the percent of microvessel area within each image (Fig. 4B). Constructs in the “light” group had the largest percentage (21.4%) of microvessel area. This was significantly greater than constructs in the “dark” group (6.8%, $p < 0.01$). Microvessel area in the “light” group was also significantly greater ($p < 0.01$) than in implanted constructs containing cells modified with only Flox_{VP}A and CRY2-Cre^N (10.1%). Constructs in the “light” group had equivalent ($p = 0.21$) microvessel area as the positive control constructs modified with CMV-Cre (16.5%). Lastly, constructs with only the

Flox_{VPA} vector and CRY2-Cre^N had equivalent levels of microvessel area when compared to the “dark” constructs ($p = 0.62$).

After euthanization, the dorsal tissue within each window chamber was harvested, and tissue extract supernatant was analyzed via ELISA for human VEGF and human ANG1 protein produced by the Flox_{VPA} cell line (Fig. 4C,D). Constructs in the “light” group showed a trend towards higher levels of VEGF than constructs in the other groups and also had significantly higher levels of ANG1 than the “dark” group ($p < 0.01$).

Discussion

In this study, we engineered a light-activated genetic “on” switch that can be used with a previously described light-inducible Cre recombinase system¹⁴ to achieve sustained transgene activation after an initial dose of illumination. This contrasts to other genetic switches, such as the tetracycline-inducible system^{47, 48}, that require the addition of a small molecule or hormone^{49, 50} to activate gene expression. Photocaged unnatural amino acids or chemical inducers have also enabled a variety of approaches to inducible transcription and recombination controlled by UV light^{12, 13, 51–54}. However, these methods currently require partial amber stop codon suppression to direct the incorporation of the photoreactive residue⁵⁵. Blue light-mediated activation eliminates any pleiotropic effects associated with an activating molecule or UV light, and it provides the opportunity to pattern gene expression by illuminating cells through a photomask (Fig. 2C,D). Another key benefit of this light-inducible system is that permanent gene activation only requires a finite exposure time to the blue light stimulus to achieve lasting gene expression. This contrasts with reversible light-inducible transcription systems^{4–11, 22}. In fact, illumination of cells for three days was sufficient to activate the system and maintain MyoD, VP64-MyoD, VEGF₁₆₅, and ANG1 protein production up to 12 days in the absence of continued light stimulus. Consequently, this tool may simplify applications such as engineering tissue constructs that contain multiple cell types, where patterns of cells are desired to express high and sustained levels of a specific factor to create an organized, heterogeneous cellular construct. In the future, these optogenetic approaches may be combined with other light-inducible strategies for controlling tissue development and vascularization, such as controlling cell adhesion and signaling⁵⁶.

We first validated the Flox vector by showing light-dependent MyoD expression in HEK293T cells and activation of the myogenic differentiation pathway in C3H10T $\frac{1}{2}$ cells. Cellular differentiation was more efficient when using CRY2-Cre^N along with CIB1^{FL}-Cre^C instead of CIB1^N-Cre^C. This is likely due to the higher recombination levels associated with CIB1^{FL}-Cre^C. Importantly, the low background levels of MyoD expression in cells treated with CRY2-Cre^N and CIB1^{FL}-Cre^C and incubated in the dark did not have a significant effect on the protein level of downstream myogenic markers (Fig. 2C–D), indicating tight control of cell differentiation.

We next showed that the genetic on switch is functional *in vivo*. We engineered a Flox vector that expresses human VEGF₁₆₅ and human ANG1 (Flox_{VPA}) in a blue-light dependent manner when co-expressed with the light-inducible Cre fragments. Cells

containing this system were immobilized in hydrogel constructs and implanted into nude mouse dorsal window chambers. Illumination of mice containing these constructs resulted in a significant increase in microvessel density when compared to similarly treated mice that were implanted with same constructs but not illuminated. Because of the activation characteristics of this system, we used CIB1^N-Cre^C instead of CIB1^{FL}-Cre^C *in vivo* in order to minimize the effects of background VEGF and/or ANG1 expression on angiogenic activity in “dark” treated mice. These results complement other recent work showing *in vivo* optogenetic control of recombination in the mouse brain to induce reporter gene expression⁵⁷. Collectively, these studies suggest this approach could be generally useful for *in vivo* regulation of gene expression in multiple tissue types to control diverse functions.

Our different approaches to construct design and delivery for the *in vitro* cell differentiation and *in vivo* tissue morphogenesis experiments highlight the need to optimize the system for the desired biological output function. In particular, the choice of CIB1^N-Cre^C or CIB1^{FL}-Cre^C, in combination with CRY2-Cre^N, depends on the application. In our experiments, CIB1^N-Cre^C in the “off” (dark) state typically yielded background levels of transgene expression equivalent to unmodified cells. Alternatively, CIB1^{FL}-Cre^C resulted in higher “off” state background expression levels of the transgene due to light-independent interactions between CRY2 and CIB1^{FL} in the dark. However, CIB1^{FL}-Cre^C also resulted in higher transgene expression levels when the genetic switch was activated. Therefore CIB1^{FL}-Cre^C may be well suited for applications like cellular differentiation that require a minimum threshold level of master transcription factor activity and seem to be unaffected by low background levels of the transgene when in the “off” state. Similarly, CIB1^{FL}-Cre^C is desirable for applications that require high activation levels of the transgene to induce the desired effect. However, systems that might be sensitive to low transgene expression levels in the “off” state, such as angiogenic sprouting, should use CIB1^N-Cre^C. Moreover, next-generation versions of the light-inducible Cre recombinase may facilitate even greater control over levels of induced recombination and downstream effects⁵⁸. Therefore the options for tight control and flexible dynamic range of the system, coupled with the ability to induce sustained effects with transient stimuli in a spatiotemporally controlled fashion, will enable many applications in tissue engineering, synthetic biology, gene therapy, and basic science.

Methods

Plasmid Construction and Cell Culture

Detailed descriptions of plasmid construction, cell culture, lentiviral preparation and transductions, plasmid transfection, and gene expression analysis are provided in the Supplementary Information.

Illumination

Cells were illuminated using a custom-built 4×6 LED array⁵⁹ (~12.5 mW, measured using a Newport Power Meter held directly above LEDs, Model No. 1931-C). Six blue Rebel LEDs (450 nm, 48 Lumens @ 700 mA, Philips Lumileds) pre-mounted on a 10 mm square base (Luxeon Star LEDs, Ontario, Canada) were connected in series, and four of these LED

series were connected in parallel and mounted on a printed circuit board (Radio Shack). The LED array was powered using an Arduino Uno microcontroller programmed to pulse illumination for 1 second at 0.067 Hz. Current was regulated using a BuckPuck AC Driver (700 mA, Luxeon Star LEDs). The LED array was held 8" above the incubator shelf with illumination facing downward such that cells were illuminated from the top of the dish. Plates containing cells incubated in the dark were wrapped in aluminum foil.

For the *in vivo* experiment, window chambers of mice containing light-sensitive constructs were either wrapped in an opaque foam sheet to shield the construct from light or were illuminated with pulsing blue light (1 second at 0.067 Hz). The 4×6 LED array was placed atop or on the side of the outside of the cage of illuminated mice, and the cage and LED array was wrapped in foil. Mice were illuminated with pulsing blue light for 8 continuous hours on the day of surgery and then 12 hours each day for the next three days.

Western Blot

Cells in Figure S1 were lysed in RIPA buffer (Sigma R0278) containing protease inhibitor (Fisher 87785). Lysates were mixed with loading buffer and boiled for 5 min; equal volumes of protein were run in a NuPAGE Novex 4–12% Bis-Tris polyacrylamide gel and transferred to a nitrocellulose membrane. Non-specific antibody binding was blocked with 5% non-fat milk in TBS-T (50 mM Tris, 150 mM NaCl, and 0.1% Tween-20) for 30 min at RT. Membrane was then incubated with an anti-MyoD primary antibody (Santa Cruz sc-32758, 1:200) in 5% milk in TBS-T overnight at 4°C and then a goat anti-mouse IgG-HRP conjugated secondary antibody (Santa Cruz sc-2005, 1:1000) in 5% milk in TBS-T for 1 hr at RT. Membrane was visualized using the Immun-Star™ WesternC™ Chemiluminescence Kit (Bio-Rad, 170-5070) according to manufacturer's directions, and the image was captured using a ChemiDoc XRS+ System and processed using ImageLab software (Bio-Rad). After image capture, the membrane was stripped of the primary and secondary antibodies using Restore PLUS Western blot stripping buffer (Thermo Scientific, 46430) for 10 minutes before blocking in 5% milk in TBS-T for 30 min at RT. Membrane was then probed with an anti-GAPDH antibody (Cell Signaling 2118L, 1:5000) in 5% milk in TBS-T for 1 hr at RT and then the anti-rabbit/HRP conjugated secondary antibody for 1 hr at RT.

Immunofluorescence staining

Cells were washed twice with PBS, fixed with 4% paraformaldehyde for 10–20 min, and then incubated in blocking buffer (2.5 g BSA, 12.5 mL FBS, 250 µl Triton-X, and PBS to a total volume of 250 mL) for 1 hr at room temperature. Cells were incubated with a Myogenin primary antibody (Santa Cruz sc-12732) diluted 1:200 in blocking buffer overnight on a rocker at 4°C, washed 2x with PBS and then incubated with a secondary antibody (Goat anti-Rabbit IgG (H+L) Alexa Fluor® 647 conjugate from Invitrogen, A-21244) diluted 1:200 in blocking buffer for 1 hr at room temperature. Cells were then washed twice with PBS and incubated with an Alexa Fluor 488-conjugated MF20 antibody overnight (MF20 antibody from eBioscience 53-6503-82) and then washed twice with PBS the following day. Finally, cells were stained with DAPI diluted 1:5000 in PBS for 10–20 min. Images were acquired using a Zeiss Axio Observer fluorescence microscope at 10x

magnification. Exposure times were kept constant across all samples within the same experiment. Contrast and brightness were adjusted equally for all images using FIJI ImageJ 1.48r.

ELISA

For *in vitro* experiments, cell culture supernatant was collected, snap-frozen in liquid nitrogen, and stored at -80°C until the assay was performed. Samples were diluted 1:10, 1:100, or 1:1000 in PBS before being loaded onto the ELISA array. Human ANG1 was detected using the Human ANGPT1 ELISA kit from RayBiotech (ELH-Angiopoietin1). Human VEGF was detected using the VEGF Human ELISA Kit from Invitrogen (KHG0112). Both ELISA kits were used according to manufacturer's directions.

For *in vivo* experiments, mice were euthanized and the dorsal flesh of each window chamber was excised and cut into four quarters. Each quarter was snap-frozen in a separate tube using liquid nitrogen and stored at -80°C . Tissues were then thawed on ice and mechanically homogenized using disposable plastic pestles (VWR, 47747-358). Mechanically homogenized tissues were then lysed and suspended in 500 μl RIPA buffer (Sigma R0278) containing protease inhibitor (Fisher 87785), vortexed, and incubated on ice for 1 hr and then centrifuged at 10,000 $\times g$ for 20 min at 4°C . Tissue lysate supernatant was then transferred to a new tube, snap-frozen in liquid nitrogen, and stored at -80°C until assayed via ELISA. When assayed, lysates were thawed on ice, and total protein concentration for each sample was determined by BCA assay. 2 or 10 μg of protein was loaded into each VEGF or ANG1 ELISA well, respectively, and each ELISA was performed according to manufacturer's directions. Each sample (4 samples/mouse due to cutting each window chamber tissue into quarters) was run in technical duplicate, and concentrations for each technical duplicate were averaged.

Cell/Hydrogel Constructs

To prepare cells for implantation, HEK293T cells that had been transduced and selected for integration of Flox_{VPA} were plated in 6-well plates at a density of 800,000 cells/well two days before making the constructs. Cells were transfected the next day using Lipofectamine 3000 (Invitrogen) according to the manufacturer's directions. For the negative control group, cells were transfected with 1.565 and 0.935 μg of empty plasmid and CRY2-Cre^N; for the positive CMV-Cre condition, cells were transfected with 1.87 μg CMV-Cre plasmid and 0.63 μg empty plasmid; for the "light" and "dark" conditions, cells were transfected with 0.935 μg each of CRY2-Cre^N and CIB1^N-Cre^C plus 0.63 μg of empty plasmid.

Cells were trypsinized, counted, and used to make the cell/hydrogel constructs the following day. Cell/hydrogel constructs were formed within square-welled polydimethylsiloxane (PDMS) molds cast from 3D-machined Teflon masters. PDMS molds were coated with 0.2% (w/v) pluronic (Invitrogen) to prevent hydrogel adhesion. Laser-cut Cerex® frames (9 mm $\times$ 9 mm, 1 mm wide rim) were placed within the PDMS mold to enable hydrogel attachment and facilitate construct handling and implantation. The cell/hydrogel mixture was made by mixing together two tubes and pipetting the mixture immediately into the Cerex® frame within the PDMS mold. The first tube contained 1.8×10^6 HEK293T cells

suspended in 2x DMEM growth medium and 4 mg/mL of bovine fibrinogen (Sigma). The second tube contained 5 mg/mL collagen and 0.2 unit/mg thrombin (Sigma). Total pipetted volume was ~110 μ l per cell construct. Cell/hydrogel constructs were placed in an incubator and allowed to gel for ~4 hours. Growth media (DMEM + 10% FBS + 1% penicillin/streptomycin) was then added to the cell constructs. The next day, cell/hydrogel constructs were removed from the PDMS molds and placed in wells of a 24-well plate containing growth media until they were implanted into window chambers that same day. Constructs that contained light-sensitive cells were created and implanted under a red safelight to avoid unwanted activation during gel preparation and surgery.

Mouse Dorsal Window Chamber Installation

All animal experiments were approved by the Duke University IACUC. Female nude mice (Charles River, ~10 wk of age) were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Dorsal window chambers were installed as previously described⁶⁰. Briefly, using aseptic technique, the dorsal skin was attached to a temporary “C-frame” at the center of the back. The skin was perforated in three locations to accommodate the screws of the chamber, and a circular region (~12 mm) of the forward-facing skin (i.e., cutis, subcutis, retractor and panniculus carnosus muscles, and associated fascia) was dissected away to accommodate the window proper. The forward and rearward pieces of the titanium dorsal skinfold chamber were assembled together from opposite sides of the skin, and a Cerex® frame with cell/hydrogel constructs was laid within the window chamber atop the rearward-facing skin. A sterile cover glass was placed over the window and cell/hydrogel construct while superfusing with sterile saline solution. The chamber was then secured with suture and the “C-frame” was removed.

Live Intravital Imaging of Blood Vessels

Intravital recordings were performed in anesthetized mice on day 7 post-surgery. Mice were anesthetized by nose cone inhalation of isoflurane and positioned on a heating pad under a microscope objective. Hyperspectral brightfield image sequences (10 nm increments from 500 – 600 nm) were captured at 5x magnification using a tunable filter (Cambridge Research & Instrumentation, Inc.) and a DVC camera (ThorLabs), as previously described⁶⁰. A custom MATLAB (MathWorks) script was applied to create maps of total hemoglobin concentration^{60, 61}. Obtained maps were further processed using local contrast enhancement in ImageJ (FIJI) and thresholded to binary images to identify vessel area and calculate the area of microvessel density within each image.

Statistical Analysis

Data is shown as mean $\pm$ standard deviation, with sample numbers indicated in figure legends. *In vitro* qualitative images shown are representative of at least two experiments performed in triplicate. Statistical significance was determined by performing a multi-way ANOVA test. Differences with $p < 0.05$ were considered significant. Tukey’s honest statistical difference test was used. Statistical analysis was performed using JMP® Pro 11.

For ELISA data of the *in vivo* experiment, the four output concentrations from each mouse were pooled and each mouse was treated as one replicate. For microvessel density data, at

least 4 images were quantified from each mouse from randomly selected spots around the window chamber. Quantified images from each mouse were pooled, and the average microvessel density per mouse was used for statistical analysis. Statistical significance was determined by Tukey's Honest Statistical Difference test with $p < 0.05$ considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Light-inducible Cre recombinase fusion proteins. CRY2- is fused to the N-terminus of CRE (CRY2-Cre^N), and either the full-length or N-terminal fragment of CIB1 is fused to the C-terminus of Cre (CIB1^{FL}-Cre^C and CIB1^N-Cre^C, respectively). (B) In the dark, CRY2 and CIB1 are not bound and Cre is inactive. Blue light initiates heterodimerization of CRY2 and CIB1, which reconstitutes the active Cre recombinase. (C) Lentiviral light-activated genetic "on" switch vector. The GFP-IRES-Puromycin resistance (Puro^r) cassette is flanked by LoxP sites and is located downstream of a constitutive human Ubiquitin C (hUbC) promoter. Any gene of interest can be cloned downstream of the second LoxP site, but the start codon is located upstream of the first LoxP site. Thus, in the absence of Cre recombinase, only translation of the GFP and Puromycin proteins occurs. Upon Cre recombinase-mediated excision of the LoxP-flanked cassette, the promoter and ATG start codon are positioned directly upstream of the gene of interest, resulting in its translation.

Figure 2.

Light-inducible myogenesis in multipotent C3H10T $\frac{1}{2}$ cells. Cells were transduced with Flox_{VM} and LV_{hUbC}-CIB1^{FL}-Cre^C, transfected with an empty plasmid, CMV-Cre, or LV_{hUbC}-CRY2-Cre^N, and incubated under pulsing blue light or in the dark. (A,B) Expression of (A) myogenic markers and (B) the recombined mRNA transcript by qRT-PCR. (mean $\pm$ SD, n = 3). Conditions not connected by the same letter are significantly different as determined by Tukey's HSD Test ($p < 0.0001$). (C) Immunofluorescence staining for co-localization of myogenin (red) and Myosin II Heavy Chain (MHC, green). Blue = DAPI, scale bar = 200 μ m for all images. Images are representative of triplicate samples. (D) Cells transduced with Flox_{VM} and LV_{hUbC}-CIB1^{FL}-Cre^C and transfected with LV_{hUbC}-CRY2-Cre^N were illuminated through a photomask (light blue shading within dotted lines on schematic). Immunofluorescence staining showed a significantly higher density of myogenin- and MHC-positive cells within the illuminated region than within the regions shielded from light (mean $\pm$ SD, n = 7–14 regions across 3 independent samples; $p < 0.001$). Conditions not connected by the same letter are significantly different as determined by Tukey's HSD Test. AB, AC: $p < 0.0001$; BC: $p < 0.05$ (E) Representative images used to generate data shown in (D). In first three greyscale images, cells are shown in black. In the merged image, myogenin is shown in red and MHCII in blue.

Figure 3.

Validation of the Flox_{VPA} lentiviral vector. (A) Cells expressing Flox_{VPA} and the light-inducible CRE constructs perform as a permanent light-inducible “on” switch. HEK293T cells were transduced with Flox_{VPA} and transfected with an empty plasmid (Flox Only), a plasmid that constitutively expresses Cre recombinase (CMV-Cre), CRY2-Cre^N and CIB1^N-Cre^C (CIB1^N Light and CIB1^N Dark), or CRY2-Cre^N and CIB1^{FL}-Cre^C (CIB1^{FL} Light and CIB1^{FL} Dark). After 24 hours, cells were either illuminated for three days and then incubated in the dark for 9 days (Light) or incubated in the dark for the entire experiment (Dark). Unmodified cells that were transfected only with an empty plasmid served as a negative control (Mock). VEGF (top panel) and ANG1 (bottom panel) expression was determined by ELISA. (B) qRT-PCR was used to detect the levels of recombination of Flox_{VPA} using primers specific for the recombined mRNA. Samples were harvested after two (blue) and seven (red) days of illumination. Conditions not connected by the same letter are significantly different as determined by Tukey’s HSD test. BD, DE, CD, BC: $p < 0.05$; all other combinations, $p < 0.01$. mean $\pm$ SD, $n=2$ biological replicates.

Figure 4. Light-inducible *in vivo* angiogenesis. (A) Live intravital imaging of mouse dorsal window chambers. Window chambers were implanted with hydrogel constructs containing HEK293T cells transduced with Flox_{VP}A and transfected with CRY2-Cre^N (Flox_{VP}A + CRY2), a plasmid that constitutively expresses Cre recombinase (Flox_{VP}A + CMV-Cre), or CRY2-Cre^N and CIB1^N-Cre^C. Mice were illuminated with pulsing blue light (Flox_{VP}A + CRY2 + Cre^N, Light) or housed under normal conditions but containing window chambers shielded from light by an attached foam sheet (Flox_{VP}A + CRY2 + Cre^N, Dark). Intravital images were analyzed for total hemoglobin concentration (top colored image) and then converted to a binary black and white image showing absence and presence of hemoglobin, respectively, within each image (bottom image). Representative figures of images having the lowest and highest microvessel area percent for each condition are shown. (B) The area of each image that contained microvessels was quantified to determine the percent of microvessel area. Conditions not connected by the same letter are statistically different as determined by Tukey's HSD test. AB, AC: $p < 0.01$; BC: $p < 0.05$. mean $\pm$ SD, $n = 3, 4, 3$, and 4 mice for Flox_{VP}A + CRY2, Flox_{VP}A + CRY2 + CMV-Cre, Flox_{VP}A + CRY2 + Cre^N Dark, and Flox_{VP}A + CRY2 + Cre^N Light, respectively. (C-D). The levels of (C) human VEGF and (D) human ANG1 within the dorsal tissue lysate was analyzed by ELISA. Black data points show individual measurements from each mouse. Conditions not connected by the same letter are statistically different as determined by Tukey's HSD test. AB: $p < 0.01$. Sample sizes are same as in (B).