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Controlling transcription in human pluripotent stem cells using CRISPR-effectors

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Abstract

The ability to manipulate transcription in human pluripotent stem cells (hPSCs) is fundamental for the discovery of key genes and mechanisms governing cellular state and differentiation. Recently developed CRISPR-effector systems provide a systematic approach to rapidly test gene function in mammalian cells, including hPSCs. In this review, we discuss recent advances in CRISPR-effector technologies that have been employed to control transcription through gene activation, gene repression, and epigenome engineering. We describe an application of CRISPR-effector mediated transcriptional regulation in hPSCs by targeting a synthetic promoter driving a GFP transgene, demonstrating the ease and effectiveness of CRISPR-effector mediated transcriptional regulation in hPSCs.

Keywords

Human pluripotent stem cells; CRISPR; Cas9; transcriptional regulation; dCas9-effectors

1. Introduction / Review

1.1 Human pluripotent stem cells

Deciphering human development and disease requires knowledge of the mechanisms regulating cellular differentiation and function. Human pluripotent stem cells (hPSCs) have the ability to self-renew *in vitro* and the potential to differentiate into any cell type of the body. These properties make hPSCs an effective model system to study factors and mechanisms governing the development of specific cell lineages and their impact on disease onset and progression. The ability to control transcription of specific genes in hPSCs allows for interrogation of the key factors necessary to maintain a given cellular state or drive cellular differentiation. Recently developed CRISPR (clustered regularly interspaced short palindromic repeats)-associated (Cas) systems have been adapted to regulate gene expression. These versatile systems allow rapid examination of gene function in human cells

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and have the potential to allow high-throughput analyses of either gene activation or repression in hPSCs and their differentiation intermediates.

1.2 CRISPR/Cas9-effector system

CRISPR systems are RNA-guided bacterial adaptive immune responses that detect and silence foreign DNA [1–4]. These systems involve recruitment and binding of a CRISPR-associated endonuclease to a specific target DNA sequence via a trans-activating CRISPR RNA (tracrRNA) and a CRISPR-derived RNA (crRNA). The protospacer, a sequence element of the crRNA, confers site-specificity through complementary base pairing, ensuring proper targeting to foreign DNA. The endonuclease then introduces double strand breaks in the target sequence [2, 3, 5].

The type II CRISPR system has been adapted as a tool for gene editing in mammalian systems. Combination of the tracrRNA and crRNA elements into one chimeric single guide RNA (sgRNA) can efficiently target Cas9 to specific DNA target sites [5–7]. This two component system (Cas9 and sgRNA) has been used to introduce double strand breaks in order to generate disease-relevant mutations or to insert donor sequences at target sites through homologous recombination [6, 7]. In addition, CRISPR nucleases have successfully down-regulated target gene expression by introducing mutations or excising an enhancer [8, 9]. However, nuclease-mediated modifications are irreversible, may require in depth knowledge of the element, can be inefficient because gene disruption relies on indel introduction caused by non-homologous end joining repair errors, may disrupt local genomic architecture, and are not adaptable for gain-of-function approaches.

To adapt CRISPR/Cas9 for gene regulation studies, the nuclease activity of Cas9 has been inactivated through mutation of catalytic residues within its nuclease domains, resulting in a nuclease dead Cas9 (dCas9) [5, 10]. Coupling dCas9 to effector domains converts the CRISPR/Cas9 technology into a site-specific programmable system, which through fused effector domains can regulate gene expression (Figure 1) [11–19]. This versatile system of transcriptional control allows for characterization of the key factors and mechanisms governing a given cellular state. The ability to multiplex sgRNAs allows for synergistic effects on gene expression and permits targeting of multiple genes simultaneously [11, 12, 14, 16, 20]. In addition, the possibility of inducing dCas9-effector or sgRNA expression may allow for temporal and spatial manipulation of gene function. When applied to hPSCs, CRISPR/dCas9-effector systems can be used to influence cellular states of differentiation intermediates and can help to dissect the individual contribution of factors to development and disease.

1.3 dCas9-effector mediated transcriptional activation

dCas9/sgRNA complexes can be modified to activate gene expression in mammalian systems when targeted upstream of endogenous transcriptional start sites. Fusion of dCas9 to the p65 activation domain or to tandem repeats of the herpes simplex virus trans-activation domain VP16 (VP48, VP64, VP160, VP192) has been shown to activate endogenous gene expression [11, 14]. In a subsequent study, fusion of dCas9 to the tripartite activator VP64-p65-Rta (VPR) resulted in higher levels of gene activation compared to VP64 fusion alone

[21]. The CRISPR/dCas9 system has also been adapted in other ways for site-specific transcriptional activation. dCas9 fused to the single-chain antibody binding SunTag allowed for multimerization of antibody-fused VP64 domains, amplifying gene expression compared to dCas9-VP64 [19]. Tethering copies of the MS2 bacteriophage coat protein-binding RNA stem loop to the 3'-end of a sgRNA activated gene expression when coexpressed with MS2-VP64 fusion proteins and dCas9 [20]. More recently, using CRISP-Disp, RNA domains are combined with sgRNA scaffolds, enabling site-specific recruitment of functional RNA modules, including natural lncRNAs such as *HOTTIP*, which subsequently mediate gene regulation [22]. All of these adapted dCas9 systems can effectively increase expression of target genes highlighting their potential use in gain-of-function studies.

Recruitment of transcriptional activation domains to endogenous loci in hPSCs and differentiation intermediates may be used to interrogate the effect of gene expression on cellular state. dCas9-effector mediated transcriptional activation has been demonstrated in hPSC systems in order to induce expression of endogenous genes and alter cell fate. Targeting dCas9-VP64 to the promoter of the developmentally relevant transcription factor *SOX17* induced its RNA and protein expression in hPSCs [16]. Recruitment of dCas9-VPR to *NGN2* or *NEUROD1* induced neuronal differentiation, suggesting the potential use of the system to modify cell fate through gene activation [21]. The ability to activate expression of endogenous genes with CRISPR-effector systems may also prove beneficial for imposing cell fate through activation of transcription factor expression. Targeting of dCas9 fused to two VP64 domains to the *MyoD1* locus of mouse primary fibroblasts activated endogenous myogenic genes comparably to traditional *MyoD1* overexpression methods, resulting in the conversion of fibroblasts into skeletal myocytes [23]. dCas9-VP64 can replace exogenous *Oct4* (*Pou5f1*) during reprogramming of mouse embryonic fibroblasts to induced pluripotent stem cells (iPSCs) by targeting the *Oct4* distal enhancer albeit at lower frequency than with a transcription activator-like effector (TALE) fused to VP64 [24]. In a recent study, dCas9-VP192 has replaced transgenic *OCT4* in reprogramming of human primary fibroblasts to iPSCs. Furthermore, dCas9-VP192 simultaneously targeted to *SOX17*, *FOXA2*, *PDX1*, and *NKX6.1* improved directed differentiation to pancreatic progenitor-like cells and activated downstream endogenous gene expression [25].

1.4 dCas9-effector mediated transcriptional repression

CRISPR-mediated transcriptional repression can occur when dCas9 is targeted to specific genomic sites resulting in steric hindrance of polymerase recruitment and transcriptional elongation [10]. In order to achieve higher levels of gene repression, fusion of dCas9 to various effector domains has been implemented. Repressor domains include the mSin interaction domain (SID) and the Krueppel repressor associated box (KRAB), which recruit repressive complexes in order to silence gene expression [26–28]. It has been demonstrated in mammalian cells that both dCas9-KRAB and dCas9-SID can be targeted to endogenous loci and effectively repress gene expression [15, 16, 18].

Systems to repress gene function are crucial for dissecting the individual contribution of factors that maintain a given cellular state. CRISPR-effector mediated transcriptional repression has been used in PSCs to probe the pluripotency network required to maintain the

stem cell state [16, 18]. Targeting the *OCT4* promoter region with dCas9-KRAB resulted in decreased *OCT4* expression and spontaneous differentiation of cells due to disruption of this key gene of the pluripotency network [16]. Similarly, targeting of regions upstream of the transcriptional start sites of *Oct4* and another pluripotency factor, *Tbx3*, using dCas9-KRAB in mouse embryonic stem cells (mESCs) effectively repressed their expression, resulting in spontaneous differentiation [18]. Through CRISPR/dCas9-effector fusion mediated transcriptional repression of pluripotency factors, these studies demonstrate the capability of the system to dissect the contribution of specific genes to a given cellular state. In future work, dCas9-effector fusions will likely be instrumental to understanding the gene regulatory networks of differentiation intermediates and ultimately the signals that maintain a cellular state.

1.5 dCas9-effector mediated epigenome engineering

Recently, fusion of dCas9 to various epigenetic modifiers has proven an effective tool to specifically target genomic sequences and modify local histone marks in order to study their effect on gene expression [17, 18]. This tool is useful in determining the individual contribution of genetic elements, such as enhancers, to target gene expression. Fusion of dCas9 to the catalytic core of the transcription activator acetyltransferase p300 (dCas9-p300^{CORE}) has been demonstrated to activate genes in human cells. When compared to dCas9-VP64, the dCas9-p300^{CORE} leads to higher levels of gene activation when targeting either promoter or enhancer regions of endogenous genes, including *IL1RN*, *MYOD* and *OCT4*. Importantly, a dCas9 fused with an inactive form of p300 was unable to activate target genes, indicating a crucial role of acetyltransferase activity for gene activation [17].

dCas9-effector mediated epigenetic engineering has been used as a tool to determine the individual contribution of genetic elements to gene expression. Fusion of dCas9 to the histone demethylase LSD1 (*KDM1A*), known to catalyze demethylation of H3K4 mono- and di-methylation [29], reduces endogenous gene expression in mESCs when specifically targeted to active enhancer regions of known pluripotency-associated genes, *Oct4* and *Tbx3*. Targeting LSD1 to the *Tbx3* enhancer led to loss of H3K4me2 and H3K27ac specifically at the enhancer region and caused spontaneous differentiation of mESCs [18]. These studies highlight the important role of individual enhancer elements in transcriptional regulation and suggest that dCas9-effector mediated epigenetic engineering can be used to functionally annotate genetic elements of a given cellular state. In the future, this technology may also prove useful in regulating transcription of target genes through DNA methylation or demethylation as has been demonstrated with TALE-effectors (TALE-DNMTs and TALE-TET1) [30, 31].

1.6 An example of dCas9-KRAB mediated transcriptional repression in hPSCs

Application of dCas9-effector systems to hPSCs allows elucidation of the key genes and mechanisms governing a cellular state. Here, we describe an example to interrogate gene function with a dCas9-KRAB fusion in hPSCs. Through targeting of a knockin minigene, this study examines the timing and degree of dCas9-KRAB mediated gene repression in hPSCs. An eGFP reporter construct was introduced into the AAVS1 locus of H1 hPSCs via zinc finger nuclease augmented gene targeting, resulting in H1 CAG-GFP hPSCs which

constitutively express GFP driven by CAG (hybrid of cytomegalovirus immediate early enhancer, chicken β -actin promoter and intron, rabbit β -globin splice acceptor). A doxycycline-inducible dCas9-KRAB (TRE-dCas9-KRAB) from the *Streptococcus pyogenes* (Sp) system [16] was stably transduced into H1 CAG-GFP hPSCs. Following viral transduction of individual site-specific sgRNAs and doxycycline treatment, dCas9-KRAB was targeted to the CAG promoter and GFP expression was reduced over time. Reduction of GFP expression to baseline wild type H1 hPSC levels was observed in a subset of H1 CAG-GFP cells. This study highlights the kinetics and extent of dCas9-KRAB repression on gene expression in the hPSC system.

2. Materials and methods

2.1 Maintenance of human pluripotent stem cells

H1 and H1 CAG-GFP hPSCs were maintained in mTeSR1 (Stem Cell Technologies, 05850) on Matrigel (Corning, 354277) coated plates. Cells were fed daily and split with TrypLE Express (Gibco, 12604) every 3–4 days in mTeSR1 supplemented with 10 μ M Y-27632 (Selleck Chemicals, S1049).

2.2 Generation of H1 CAG-GFP hPSCs

24 hours prior to electroporation, H1 hPSCs were treated with 10 μ M Y-27632. On the day of electroporation, cells were split to single cells with TrypLE Express. 10×10^6 H1 hPSCs were resuspended in 800 μ L EmbryoMax electroporation buffer (Millipore, ES-003-D) and incubated with 40 μ g CAG-GFP plasmid (Addgene plasmid #22212)+ 10 μ g zinc finger nuclease plasmid (targeting the AAVS1 locus, sequences obtained from [32]) for 5 min at 4C. Cells were electroporated at 250 V, 500 μ F and plated onto puromycin-resistant MEFs in hESC media (KnockOUT DMEM supplemented with 20% KnockOUT Serum Replacement, 1 $\times$ Glutamax, 1 $\times$ MEM NEAA, 55 μ M β -ME, 10 ng/mL bFGF) and 10 μ M Y-27632. 48 hours after electroporation, cells were treated with 0.5 μ g/mL puromycin (Sigma-Aldrich) daily in order to select for cells that stably integrated the CAG-GFP construct. 11–14 days following the start of selection, surviving clones were monitored for GFP expression and picked onto Matrigel-coated plates. Site-specific integration was confirmed by PCR using the following primer sequences (forward primer: GCTCTACTGGCTTCTGCG; reverse primer: AGAAGACTTCCTCTGCC). One correctly integrated clone was expanded into the H1 CAG-GFP hPSC line used in this study.

2.3 sgRNA and plasmid design

sgRNA *in silico* design was performed as previously described [16], candidate sgRNAs were identified by searching for (N)_{19–21}NGG motifs in the CAG promoter. sgRNAs were then cloned into the pLenti U6 Sp sgRNA BsmBI hygro cloning vector (Addgene plasmid #62205) to generate sgRNA lentiviral expression constructs. sgRNA target sequences are listed in table 1 and the protospacer adjacent motif (PAM) is underlined. Tips for sgRNA design can be found in the troubleshooting section.

2.4 Lentivirus production

Lentivirus was produced in HEK293T/17 cells (ATCC, CRL-11268). Briefly, pHAGE TRE-dCas9-KRAB (Addgene #50917), or sgRNA coding plasmids were transfected with 3rd generation lentiviral packaging plasmids using TransIT-293 transfection reagent (Mirus, 2700) in Opti-MEM (Gibco, 31985) according to the manufacturer's instructions. Virus was harvested 48 hours after transfection.

2.5 Generation of stable H1 CAG-GFP TRE-dCas9-KRAB cell line

H1 CAG-GFP hPSCs were split to single cells with TrypLE Express and incubated with TRE-dCas9-KRAB lentivirus for 3 hours in a low attachment plate. Transduced cells were then plated onto Matrigel-coated plates in mTeSR1 supplemented with 10 μ M Y-27632. 48 hours after transduction, cells were treated with 50 μ g/ml G418 (Gibco, 10131) in order to select for cells that were transduced with TRE-dCas9-KRAB. Following 6 days of selection, the resulting pool of G418-resistant cells were maintained on Matrigel-coated plates in mTeSR1 supplemented with 50 μ g/ml G418 and were split with TrypLE Express every 3–4 days.

2.6 Transduction of cells with CAG-specific sgRNA lentivirus

H1 CAG-GFP TRE-dCas9-KRAB hPSCs were split to single cells with TrypLE Express and incubated with CAG-specific sgRNA lentivirus for 3 hours in a low attachment plate. Transduced cells were then plated onto Matrigel-coated plates in mTeSR1 supplemented with 10 μ M Y-27632 and 50 μ g/ml G418. 48 hours after transduction, cells were treated with 25 μ g/ml hygromycin B (Invitrogen, 10687010) in order to select for cells that were transduced with CAG-specific sgRNAs. Following 3 days of selection through completion of the experiment, the media was additionally supplemented with 2 μ g/ml doxycycline. The media was changed daily and the cells were split with TrypLE Express every 4 days. Cells were monitored for GFP expression daily.

2.7 Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and then incubated with 5% donkey serum (Jackson ImmunoResearch, 017-000-121) in PBS supplemented with 0.2% Triton X-100 (PBST) for 1 hour at room temperature. Cells were washed 3 times with PBST for 5 min. Hoechst (Invitrogen, H3570) was used for nuclei staining. Fluorescence images were obtained on a Nikon Eclipse Ti microscope.

2.8 Flow cytometry

H1 and H1 CAG-GFP TRE-dCas9-KRAB cells were dissociated to single cells with TrypLE Express, centrifuged at $300 \times g$ for 5 min, and resuspended in flow cytometry staining buffer (PBS + 2% FBS). Data were collected on a BD Accuri C6 flow cytometer daily throughout the time-course and analyzed using FlowJo version 10. Dead cells were excluded from analysis with 7-AAD Viability Staining Solution (eBioscience, 00-6993-50).

3. Results and Discussion

3.1 Constitutive GFP expression in H1 CAG-GFP hPSCs

Using a zinc finger nuclease targeting method, a CAG promoter driving GFP expression was introduced into the AAVS1 locus of H1 hPSCs as described previously (Figure 2A) [32]. AAVS1 is a ‘safe-harbor’ locus in human cells that has an open chromatin structure enabling site-specific integration of the CAG-GFP construct [32, 33]. The CAG-GFP targeting vector included a puromycin-resistance cassette, allowing for selection of targeted clones. Following puromycin selection, GFP positive hPSC colonies were picked and site-specific integration was confirmed by PCR. One clone was expanded into the H1 CAG-GFP hPSC line, maintaining hPSC colony morphology and constitutively expressing GFP (Figure 2B).

3.2 dCas9-KRAB-mediated transcriptional repression of GFP in H1 CAG-GFP hPSCs

Using the H1 CAG-GFP hPSCs, we sought to determine if dCas9-KRAB could effectively repress expression of the constitutive GFP transgene. In order to temporally control dCas9-KRAB expression, we virally introduced a doxycycline-inducible dCas9-KRAB harboring a neomycin-resistance cassette into H1 CAG-GFP cells (Figure 2C) and selected with G418 for cells that were stably transduced. In order to determine whether recruitment of dCas9-KRAB to the CAG promoter could reduce GFP expression, we individually transduced four different CAG-specific sgRNAs that target regions of the CAG promoter (Figure 2A). Through the presence of a hygromycin-resistance cassette in the sgRNA constructs, we were able to select for cells that were transduced with sgRNA virus by treating with hygromycin B.

Doxycycline was supplemented daily in order to induce dCas9-KRAB expression in the cells. After 9 days of doxycycline and selection reagent treatment, fluorescence analysis revealed reduction in the GFP expression of a subset of H1 CAG-GFP cells transduced with sgRNA C and to a lesser extent with sgRNAs A, B, and D as compared to the no sgRNA control condition (Figure 2D). The differing effect observed with each sgRNA is likely due to the lack of clonality in our transduced pools of cells, resulting in heterogeneous expression of both the sgRNA and dCas9-KRAB. Flow cytometry analysis at day 9 revealed reduced GFP fluorescence levels in a subset of H1 CAG-GFP cells when transduced with each sgRNA as compared to GFP levels of the no guide control condition (Figure 2E). sgRNAs A, B, and D have stretches of the same nucleotide in their sequences which may also contribute to the differing abilities of each sgRNA to repress GFP (see section 4. Troubleshooting for sgRNA design tips).

To determine baseline GFP levels in both wildtype H1 and H1 CAG-GFP hPSCs, we co-cultured wild type H1 cells mixed with H1 CAG-GFP cells before analyzing for GFP expression by flow cytometry (Figure 2F, blue histogram). This mixture of cells shows two distinct populations, allowing us to set reference points for GFP positive and GFP negative populations in subsequent experiments.

We next sought to determine the kinetics and extent of dCas9-KRAB mediated GFP repression. We performed a representative time-course of dCas9-KRAB mediated GFP repression with sgRNA C, where reduction in GFP expression levels was analyzed daily by

flow cytometry (Figure 2F, green histograms). H1 CAG-GFP TRE-dCas9-KRAB cells were transduced with sgRNA C and GFP fluorescence levels were analyzed daily following the start of doxycycline treatment. GFP levels were reduced in a subset of cells by day 3 compared to the no sgRNA guide control, and continued to decrease toward the GFP negative reference point daily. GFP levels decreased to the GFP negative reference point by day 10, indicating the ability of the system to completely repress GFP in a subset of cells. As we are repressing transcription of the CAG-GFP transgene, reduction in GFP expression levels is limited by the stable half-life of the GFP protein, which may explain the gradual decrease in GFP levels [34, 35]. It is likely that protein reduction may occur faster when repressing genes that encode less stable proteins. Using this platform, we have demonstrated that targeting a transgene expressed in hPSCs with dCas9-KRAB can reduce target protein to baseline levels and established a time-course for dCas9-KRAB to achieve complete repression as measured by protein levels.

3.3 Summary

CRISPR-effector systems provide the ability to manipulate gene expression in hPSCs and thereby study gene function. We demonstrate that dCas9-KRAB can completely repress gene expression in H1 CAG-GFP hPSCs. The ability to influence gene expression in a given cellular state will provide vital information on signals and factors necessary to maintain or transition from that state. The CRISPR-effector system provides a highly rapid and versatile tool to dissect the cell regulatory networks crucial for cell fate decisions. When applied to hPSC differentiation studies, these systems of transcriptional control will be instrumental in determining the key genes linked to cellular state and those that enable differentiation toward clinically-relevant, mature cell types.

4. Troubleshooting

4.1 sgRNA design

An sgRNA for the Sp system contains both a 20-nucleotide DNA targeting sequence and a Cas9 recruiting sequence. The DNA targeting sequence consists of the 20 nucleotides immediately 5' to the genomic PAM sequence (NGG). While studies examining the effects of sgRNA design on dCas9-effector activity are limited, recent studies using Sp Cas9 nuclease have determined more optimal sgRNA design parameters for CRISPR-mediated gene inactivation through screening of sgRNA pools at endogenous loci [36–38]. These studies highlight sequence features of the sgRNA and the targeted DNA that may be beneficial for sgRNA activity. First, cytosine is preferred as the variable nucleotide of the PAM sequence (CGG) [37, 38]. Guanine is preferable as the nucleotide immediately adjacent to the PAM (GNGG) [36, 37]. Avoid sgRNAs with high or low GC-content; GC-content between 40–80% is desirable [36]. Also avoid long stretches of the same nucleotide in sgRNA sequences as these may trigger RNA polymerase termination and result in disrupted transcription of the sgRNA in your system [36, 37]. Publically available software can help to design optimal sgRNA molecules, including sgRNA scorer 1.0 (crispr.med.harvard.edu/sgRNAScorer [38]) and the Broad sgRNA Designer (broadinstitute.org/rnai/public/analysis-tools/sgrna-design [37]).

Off-target genomic sequences should be examined and sgRNA molecules with high off-targeting potential should also be avoided. In order to increase specificity of the system, make sure that off-target genomic sequences are not followed by a PAM [39]. Maximize nucleotide mismatches within off-targets, avoiding sgRNA sequences that have off-target sequences with less than three mismatches and stretches of mismatches are preferred [39]. Publically available software can predict off-target sequences and calculate mismatches between sgRNAs and off-targets, including the MIT CRISPR design tool (crispr.mit.edu [39]).

4.2 Using other dCas9-effectors in the hPSC system

dCas9-effectors can be employed for transcriptional regulation in hPSCs as exemplified here through dCas9-KRAB mediated transcriptional repression, however alternative mechanisms of gene regulation can easily be achieved by utilizing dCas9 fused to alternative effectors. Many Sp dCas9-effector systems are available through principal investigators or from Addgene. We have previously demonstrated the ability of a dCas9-VP64 fusion to activate expression of *SOX17* in hPSCs [16] and others have used dCas9-VP192 to activate developmentally relevant genes in hPSCs and during differentiation [25]. These studies highlight the potential use of various dCas9-effector fusions in hPSCs. Using a variety of dCas9-effector fusions in the hPSC system will be a powerful tool for probing various cell states and altering cell fate during cellular differentiation.

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Abbreviations

CAG	hybrid of cytomegalovirus immediate early enhancer, chicken β -actin promoter and intron, rabbit β -globin splice acceptor
Cas	CRISPR-associated
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR-derived RNA
dCas9	nuclease-dead Cas9
hPSC	human pluripotent stem cell
KRAB	Krüppel repressor associated box
mESC	mouse embryonic stem cell
PAM	protospacer adjacent motif
sgRNA	single guide RNA
Sp	<i>Streptococcus pyogenes</i>

tracrRNA	trans-activating CRISPR RNA
TALE	transcription activator-like effector

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Highlights

- Applications of CRISPR-effector technology in mammalian cells and hPSCs is reviewed
- Effect of dCas9-KRAB on model locus in hPSC is investigated
- Time course of dCas9-KRAB mediated repression of GFP transgene in hPSCs is provided

Figure 1. CRISPR/Cas9 effector-mediated transcriptional control

(A) Schematic depicting dCas9-effector mediated transcriptional regulation through fusion of dCas9 to transcriptional activation or repression effector domains. (B) Schematic depicting dCas9-effector mediated transcriptional regulation through recruitment of multiple antibody-fused effector domains to a dCas9-fused epitope array. (C) Schematic depicting dCas9-effector mediated transcriptional regulation through recruitment of MS2-effector fusion proteins to tethered copies of the MS2 bacteriophage coat protein-binding RNA stem loop on the 3'-end of an sgRNA. (D) Schematic depicting dCas9-effector mediated transcriptional regulation through scaffolding of a functional RNA module (such as an aptamer or lncRNA) to an sgRNA. (E) Schematic depicting dCas9-effector mediated transcriptional regulation through fusion of dCas9 to epigenetic modifiers to alter local epigenetic marks.

Figure 2. dCas9-KRAB-mediated repression of GFP in H1 CAG-GFP hPSCs
 (A) Reporter construct inserted into the AAVS1 locus of H1 hPSCs via zinc finger nuclease gene editing. GFP expression is driven by a CAG (CMV-IE, chicken actin, rabbit beta globin) promoter. Target locations of CAG-specific sgRNAs (A, B, C, and D) and the transcriptional start site (TSS) are indicated. (B) Representative phase contrast and fluorescence images of H1 CAG-GFP cells demonstrating that the targeted cells maintain hPSC morphology and constitutively express GFP. Scale bar = 500 μ m (C) Schematic of doxycycline-inducible dCas9-KRAB and CAG-specific sgRNA lentiviral constructs. rtTA expression is constitutively driven by the Ubiquitin C promoter (UbiC) which binds the Tet-responsive element (TRE) in the presence of doxycycline in order to activate dCas9-KRAB expression. CAG-specific sgRNA expression is constitutively driven by the U6 promoter. (D) Fluorescence and (E) flow cytometry analysis of H1 CAG-GFP cells transduced with a CAG-specific sgRNA demonstrating reduced GFP expression in an sgRNA-dependent manner following 9 days of doxycycline treatment. Scale bar = 100 μ m. (F) Flow cytometry analysis of H1 CAG-GFP cells mixed with H1 cells (blue) indicating baseline fluorescence levels of both populations. Flow cytometry analysis of H1 CAG-GFP cells transduced with CAG-specific sgRNA C (green) prior to doxycycline treatment (Day 0) and acquired daily following doxycycline treatment (Day 1–Day 10). The percentage of the subset of cells that are losing GFP expression (%GFP negative) is indicated in the upper left of each time-point panel.

Table 1

CAG-specific sgRNA target sequences

sgRNA	Target sequence (PAM underlined)
A	ctccgaaagtttcctttat <u>gg</u>
B	tataaaaagcgaagcgcgcgc <u>ggg</u>
C	cgttactcccacaggtgagc <u>gg</u>
D	tgaaagccttgaggggctcc <u>gg</u>