Cas9 effector-mediated regulation of transcription and differentiation in human pluripotent stem cells

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ABSTRACT
The identification of the trans-acting factors and cis-regulatory modules that are involved in human pluripotent stem cell (hPSC) maintenance and differentiation is necessary to dissect the operating regulatory networks in these processes and thereby identify nodes where signal input will direct desired cell fate decisions in vitro or in vivo. To deconvolute these networks, we established a method to influence the differentiation state of hPSCs with a CRISPR-associated catalytically inactive dCas9 fused to an effector domain. In human embryonic stem cells, we find that the dCas9 effectors can exert positive or negative regulation on the expression of developmentally relevant genes, which can influence cell differentiation status when impinging on a key node in the regulatory network that governs the cell state. This system provides a platform for the interrogation of the underlying regulators governing specific differentiation decisions, which can then be employed to direct cellular differentiation down desired pathways.

KEY WORDS: CRISPR, Cas9, Differentiation, Gene activation, Pluripotent stem cell, Transcriptional repression

INTRODUCTION
Human pluripotent stem cells (hPSCs) offer a unique avenue to study normal as well as defective cellular differentiation and function in vitro, and have great potential to advance understanding and treatment of diseases. However, for many cell types of interest (e.g. mature pancreatic β-cells and thymic epithelial cells), our inability to guide hPSCs towards the desired mature and functional cell types through the application of exogenous signaling molecules precludes utilization of this in vitro system in many areas. Exogenous delivery of transcription factors provides an alternative method to influence cell identity in hPSCs and to elucidate regulatory networks underlying these cell fate decisions. Recently, an RNA-guided adaptive immune system that underpin hPSC differentiation.

RESULTS AND DISCUSSION
We created a lentiviral delivery-based dCas9-E/CRISPRe system to modulate gene expression in human embryonic stem cells (hESCs), using either CRISPR interference (CRISPRi) or CRISPR activation (CRISPRa). We describe the application of CRISPRi to influence directly the differentiation status of stem cells has not been addressed. Here, we demonstrate the ability of the CRISPR effector (CRISPRe) system to modulate gene expression in human embryonic stem cells (hESCs), using either CRISPR interference (CRISPRi) or CRISPR activation (CRISPRa). We describe the application of CRISPRi to influence directly the differentiation status of hESCs, providing a platform for interrogating transcriptional regulatory networks in vitro that underpin hPSC differentiation decisions.


Received 3 September 2013; Accepted 1 October 2013
repression epigenetic mark of a poised gene (Rada-Iglesias et al., 2011) (Fig. 1A). This regulator offers a unique opportunity to address whether we could activate a differentiation marker in a poised state. To assess the ability of dCas9-VP64 to upregulate expression of SOX17 in hESCs, we expressed dCas9-VP64 in hESCs under control of the ubiquitously expressed EF1α promoter and designed two sgRNAs to target regions upstream of the SOX17 transcriptional start site (TSS) (Fig. 1A,B). Neither expression of the dCas9-VP64 variant alone nor the presence of SOX17-specific sgRNAs alone led to significant increases in SOX17 expression levels. Likewise, delivery of dCas9-VP64 in conjunction with the SOX17-126 sgRNA had no detectable effect. By contrast, co-delivery of dCas9-VP64 and SOX17-177 sgRNA increased expression of SOX17 by 287±35-fold (Fig. 1C). This increase in gene expression was sufficient to allow the accumulation of SOX17 protein in the treated hESC cultures based on immunofluorescence analysis (Fig. 1D). Thus, despite the presence of repressive epigenetic marks, the CRISPRa system can drive expression of developmentally relevant genes in hESCs with one sgRNA.

To ascertain the number of hESCs responding to the CRISPRa system, we delivered SOX17-177 sgRNA or a control sgRNA into TRE-regulated dCas9-VP64 cells (supplementary material Fig. S3A). In this system, selection of the cells with neomycin and puromycin enables the combined enrichment of TRE-regulated dCas9-VP64 and each sgRNA. Combined selection was followed by 6 days of doxycycline treatment, resulting in 25.4% of cells with antibody-detectable SOX17 expression compared with 0% of control cells (supplementary material Fig. S3B,C). This variability might be due to variations in the expression levels of CRISPRa components due to the location of viral integration or subsequent silencing of the viral constructs. Variation in the underlying epigenetic state of each cell might also contribute to its overall responsiveness to the CRISPRa system.

The ability of an sgRNA to mediate dCas9-VP64-based changes in gene expression in hESCs is consistent with studies of similar dCas9 activators in transformed human cell lines (Cheng et al., 2013; Mali et al., 2013a; Maeder et al., 2013; Gilbert et al., 2013; Perez-Pinera et al., 2013). However, in these studies co-expression of multiple sgRNAs is typically required for efficient gene activation (Cheng et al., 2013; Gilbert et al., 2013; Mali et al., 2013a Perez-Pinera et al., 2013). To determine whether a combination of sgRNAs would further increase SOX17 expression through CRISPRa in hESCs, we transduced cells with SOX17-177, SOX17-126, SOX17-296 or SOX17-91 sgRNAs individually or in combination. A combination of all SOX17 sgRNAs increased SOX17 protein-specific immunoreactivity in a subset of cells compared with individual sgRNAs (supplementary material Fig. S3B,D).

Quantification of expression levels in the SOX17+ populations revealed a statistically significant increase (P<0.01) in the mean expression level of SOX17 when multiple sgRNAs were employed, consistent with an additive effect between different sgRNAs targeting the same promoter (supplementary material Fig. S3E). We conclude that, although a specific sgRNA suffices to increase gene expression in hESCs, multiple sgRNAs can further improve expression levels of a target gene.

Although the imposed activity of specific transcription factors has proven effective in the programming of cell fate decisions (reviewed by Ladewig et al., 2013), the destabilization of transcription factors that govern cell identity can also be used to change cell differentiation status (e.g. Cobaleda et al., 2007; Hay et al., 2004). To address whether our dCas9-E system can be harnessed for this purpose in hESCs, we targeted the pluripotency network by combining the dCas9-KRAB transcriptional repressor with OCT4-targeting sgRNAs. Two isoforms of OCT4 (also known as POU5F1) are expressed in hESCs; isoform A is important for maintaining pluripotency whereas isoform B does not activate transcription of OCT4-dependent promoters (Lee et al., 2006). We designed sgRNAs to target the TSS of either OCT4 isoform A or isoform B (Fig. 2A,B). Six days after co-expression of dCas9-KRAB and individual sgRNAs, we observed a more flattened morphology of cultures that contained OCT4A-specific sgRNAs compared with hESCs that received OCT4B-specific sgRNAs (Fig. 2C; supplementary material Fig. S4A). In addition, a large number of OCT4-negative cells were present in the cultures that received...
OCT4A-specific sgRNAs (supplementary material Fig. S4B), indicating the desired silencing of this locus.

To address whether dCas9-KRAB mediated repression of a pluripotency-associated gene is sufficient to influence hESC differentiation status, we analyzed the dCas9-KRAB cultures at day 6 after sgRNA delivery for expression of the pluripotency factor NANOG, as well as for differentiation markers for trophectoderm (CDX2), endoderm (SOX17) and mesendoderm/mesoderm (T). Whereas virtually every cell in the dCas9-KRAB cultures with a control sgRNA expressed both OCT4 and NANOG, large areas of OCT4 and NANOG-negative cells were observed with OCT4A-specific sgRNAs (Fig. 2D; supplementary material Fig. S4C). The expression of the dCas9-KRAB variant with a control sgRNA did not influence OCT4 expression or colony morphology, suggesting that the dCas9-E/CRISPRe components are not negatively influencing self-renewal independent of their gene-specific targeting function. We conclude that the dCas9-E/CRISPRe system is able to influence hESC differentiation with one sgRNA.

Recently, it was reported that a CRISPRi system can be implemented by steric interference with RNA Polymerase II transcriptional initiation or elongation via dCas9 binding (Qi et al., 2013; Gilbert et al., 2013). To address whether the observed effects of dCas9-KRAB on hESCs in our system were due to an obstruction mechanism rather than KRAB-mediated repression, we tested the impact of a dCas9 variant lacking the KRAB effector domain on OCT4 expression. We did not observe any morphological changes in hESC cultures co-expressing dCas9 and the OCT4A sgRNA targeting the −158 region upstream of the TSS (supplementary material Fig. S4D).
material Fig. S4D). Interestingly, we found rare cells with morphological changes in the cultures co-expressing dCas9 and the OCT4A sgRNA proximal to the TSS (OCT4A-12; supplementary material Fig. S4D). To address whether the morphological changes were accompanied by a change in expression of pluripotency genes or differentiation-associated factors, we analyzed the cultures for the presence of OCT4 and NANOG, or CDX2, SOX17 and T, respectively. In concordance with the subtle morphological changes, we observed patches of OCT4 and NANOG downregulation in hESC dCas9 cultures containing the OCT4A-12 sgRNA, but not in cultures containing the OCT4A-158 sgRNA (Fig. 2E). In addition, SOX17 antibody-reactive cells were detected within the NANOG-downregulated regions of the dCas9/OCT4A-12 sgRNA-treated cultures. Overall, the impact of the effector-less dCas9 on differentiation-associated genes is attenuated relative to dCas9-KRAB. Thus, some degree of repression associated with the dCas9-KRAB/OCT4A-12 sgRNA combination is likely to be due to direct interference of dCas9 with the function of RNA Polymerase II, whereas the repression associated with the dCas9-KRAB/OCT4A-158 sgRNA combination is dependent on the KRAB effector domain.

To address whether the effector-independent dCas9 might impose a delayed effect on differentiation relative to the dCas9-KRAB effector, we analyzed the appearance of differentiation-associated transcripts in a time course for both versions of dCas9 (Fig. 2F,G). No significant increases in CDX2, T, SOX17, SOX7 or AFP transcripts were detected in dCas9/OCT4A sgRNA-treated cultures over the course of 12 days. By contrast, dCas9-KRAB/OCT4A-158 sgRNA-treated cultures showed increases in CDX2, SOX17 and T starting at day 3, in SOX7 at day 6 and in AFP at day 12, consistent with the differentiation of a fraction of the treated cells down various developmental pathways. Cells treated with dCas9-KRAB/OCT4A-12 sgRNA displayed similar expression of differentiation markers (Fig. 2G).

This proof-of-principle study demonstrates the potency of dCas9-E/CRISSPre for the activation or repression of key transcription factors in hPSCs that can have dramatic effects on gene expression and differentiation status. We suggest that this approach will have wide applicability in altering gene expression to modulate cell fate decisions in various stem cell populations. These experiments could be performed in a directed manner, or using library-based lentiviral approaches similar to those employed with shRNA libraries (Moffat et al., 2006; Kagey et al., 2010). We envisage that this system will be instrumental in dissecting regulatory networks in hPSC derivatives and thereby improving our understanding of their contribution to development or disease.

MATERIALS AND METHODS

sgRNA in silico design

Candidate sgRNAs were identified by searching for G(N)20GG motifs 300 bases upstream and 100 bases downstream of the TSS that conform with the nucleotide requirements for U6 Pol III transcription and the spCas9 PAM recognition element (NGG) (Jinek et al., 2012; Mali et al., 2013b). Bowtie2 was used to map candidate targets to the human genome (build GRCh37) (Langmead and Salzberg, 2012) with sensitive parameters (−local -f-k 10 --very-sensitive-local -L 9 -N 1) to detect potential off-target sites (supplementary material Table S1). All our sgRNAs had no other genomic matches at the alignment stringency used.

Plasmid design and construction

The human codon-optimized, nuclease-deficient Cas9 [dCas9; D10A, H840A (Jinek et al., 2012; Qi et al., 2013)] was generated by gene synthesis (GenScript). The KRAB repressor domain [residues 1-75 from ZFN10 (Homo sapiens)] (Cong et al., 2012), Addgene 42945] was subcloned to the 3′-end of the dCas9-NLS-3xHA to generate the dCas9-KRAB lentiviral expression construct. The VP64 activation domain [from Addgene 32188 (Zhang et al., 2011)] was subcloned to the 3′-end of the dCas9-NLS-3xHA to generate the dCas9-VP64 lentiviral expression construct. The sgRNA expression lentiviral vector is based on the plKLO1 plasmid with an oligonucleotide cloning site containing two BfuAI sites for inserting guide sequences via 4-bp 5′ overhangs (ACCG and AAAC) into the sgRNA sequence (Jinek et al., 2012) (supplementary material Fig. S1).

Virus production

HEK293T/17 cells were maintained in Dulbecco’s Modified Eagle Medium (Gibco, 11965) supplemented with 10% fetal bovine serum (Valley Biomedical, BS3033) and Glutamax (Gibco, 03505). HEK293T/17 cells were split and plated at 1.3×10^6 cells/cm^2. The following day, packaging plasmids and dCas9-, dCas9-E- or sgRNA-coding plasmids were transfected 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.

Human embryonic stem cell culture

H1 cell lines were maintained on Matrigel (BD Biosciences, 354277) in mTeSR1 (Stem Cell Technologies, 05850). TRE-regulated dCas9 or dCas9-E lines were supplemented with 100 ng/ml geneticin (Gibco, 10131) and EF1α-regulated dCas9-E lines with 1 μg/ml puromycin (Sigma-Aldrich). Cells were fed daily and split every 3-4 days with TrypLE Express (Gibco, 12604) in the presence of 10 μM Y-27632 (Selleck Chemicals, S1049).

Generation of stable dCas9/dCas9-E cell lines and co-expression with sgRNAs

H1 cells were washed with PBS and singularized with TrypLE Express. Cells were incubated with TRE-regulated or EF1α-regulated dCas9 or dCas9-E lentivirus on low attachment plates. After 3 hours, cells were plated onto Matrigel-coated plates with 10 μM Y-27632. From 48 hours after transduction, EF1α-regulated dCas9-E transduced cells were treated with 1 μg/ml puromycin and TRE-regulated dCas9 or dCas9-E transduced cells with 100 ng/ml geneticin to select and maintain stable cell lines. For experiments utilizing sgRNAs, the appropriate stable dCas9 or dCas9-E cell lines were incubated with sgRNA lentiviruses as above and plated at 1.25×10^5 cells/cm^2. Forty-eight hours after transduction, TRE-regulated dCas9 or dCas9-E sgRNA transduced cells were treated with 1 μg/ml puromycin to select for cells expressing the sgRNA and 2 μg/ml doxycycline (Sigma-Aldrich) to induce expression of dCas9 or dCas9-E (day 0). An sgRNA targeting the CAG (CMV-IE, rabbit beta globin) promoter was used as an off-target control.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature then blocked for 45 minutes with 5% donkey serum (Jackson ImmunoResearch, 017-000-121) in PBST [PBS + 0.2% Triton X-100 (Sigma-Aldrich)]. Cells were incubated with primary antibodies in blocking buffer (supplementary material Table S2) for 3 hours at room temperature, then washed three times with PBST. Cells were incubated with Alexa-Fluor-conjugated secondary antibodies (Invitrogen, 1:300) for 2 hours at room temperature and washed three times with PBST. Nuclei were stained with Hoechst (Invitrogen, H3570). Phase contrast images were acquired on Nikon Eclipse TS100 and fluorescent images on Nikon Eclipse Ti microscopes. Cells were quantified using NIS-Elements Analysis Software. Ten random fields at 20× magnification were counted (3400-5000 cells identified through Hoechst staining) and the mean fluorescence intensity of SOX17 signal in each cell calculated based on a threshold set using the CAG-sgRNA control.

Quantitative PCR analysis

RNA was isolated using Trizol Reagent (Invitrogen, 15596-018) according to the manufacturer’s instructions. Total RNA (2 μg for SOX17 analysis, or 250 ng for OCT4 analysis) was reverse-transcribed using SuperScript III
First-Strand Synthesis System (Invitrogen, 18080-051). cDNA (30 ng for SOX17 analysis, or 3.75 ng for OCT4 analysis) was utilized in qPCR reactions using specific primers listed in supplementary material Table S3 in iTAQ Universal SYBR Green Supermix (Bio-Rad, 172-5124) or by the following TaqMan assays: ACTB (Hs01060665_g1), SOX17 (Hs00751752_s1). Relative gene expression was calculated using the ΔΔCT method; all genes were normalized to ACTB.

**Epigenetic data analysis**

Fig. 1A and Fig. 2A were generated from the Integrative Genome Viewer (IGV) (Robinson et al., 2011) using the publicly available ENCODE epigenetic sequence data for the human H1 cell line.

**Acknowledgements**

We thank Dr Michael Green (UMass Medical School), and Drs Guang Hu and Stephen Elledge (Harvard Medical School) for plasmids.

**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

R.M. and S.A.W. developed the concepts and approach, performed experiments and data analysis. N.A.K., R.M.J.G. and M.S.E. developed the approach, performed experiments and data analysis. M.G. developed the sgRNA target analysis approach. All authors prepared and edited the manuscript prior to submission.

**Funding**

This research was supported by the US National Institutes of Health (NIH [R01GM068110 to S.A.W.]; and by the Helmsley Charitable Trust and the Glass Family Charitable Foundation (R.M.). Deposited in PMC for release after 12 months.

**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.103341/-/DC1

**References**


*Development (2014) doi:10.1242/dev.103341*