

CRISPR gene editing and transcriptional activation in human iPS cells

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Introduction

The CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated 9) gene editing system is a powerful tool for creating mutations and insertions in a cell's genomic DNA^(1,2,3). Co-delivery of a Cas9 nuclease and a DNA-targeting guide RNA causes a targeted double-strand break, prompting the cellular machinery for repair. Researchers have been able to expand the capability of the CRISPR-Cas9 system by including a repair template for use with homology-directed repair. Additionally, an inactive, also known as a dead, Cas9 nuclease has been demonstrated to inhibit or activate gene expression when fused to an effector domain^(4,5,6). The power behind CRISPR-Cas9 is the ease of gene editing reagent construction and the ability to use this technology in many different types of cells.

Induced pluripotent stem cells are a valuable research tool for ability to be turned into virtually any cell type. With the advent of CRISPR-Cas9 researchers can modify the genome of the iPS cells and subsequently differentiate them to see the affect that the genomic modification has upon the differentiated cell type. Here we present the ability to modify the genome and modulate expression of genes within a human induced pluripotent stem (iPS) cell line with Dharmacon™ Edit-R CRISPR and CRISPRmod CRISPRa reagents. We demonstrate the following:

- Performing gene editing with Edit-R Cas9 nuclease and synthetic RNA
- Knockout of *POU5F1*(*OCT4*) to induce iPS cell differentiation determined via live-cell fluorescent microscopy
- CRISPRa mediated activation of gene transcription with CRISPRmod CRISPRa dCas9-VPR nuclease and synthetic RNA
- HDR mediated knock-in of EGFP to create an EGFP tagged protein

Results

Gene editing with Edit-R Cas9 nuclease and synthetic guide RNA

A human induced pluripotent stem (iPS) cell line was grown in a feeder cell-free culture environment and was validated to be undifferentiated via live-cell fluorescent microscopy before plating for transfection (Figure 1A). Undifferentiated iPS cells were disassociated and transfected using the Lonza Nucleofector with Edit-R Cas9 Nuclease protein, synthetic crRNA, and synthetic tracrRNA targeting the human *PP1B* gene. After nucleofection, cells were plated into a 6-well dish and placed into a 37 °C incubator for an additional forty-eight hours. A DNA mismatch detection assay (T7 endonuclease I; T7EI) was performed and a 31% indel (insertions or deletions) rate was observed for the *PP1B* gene target (Figure 1B).

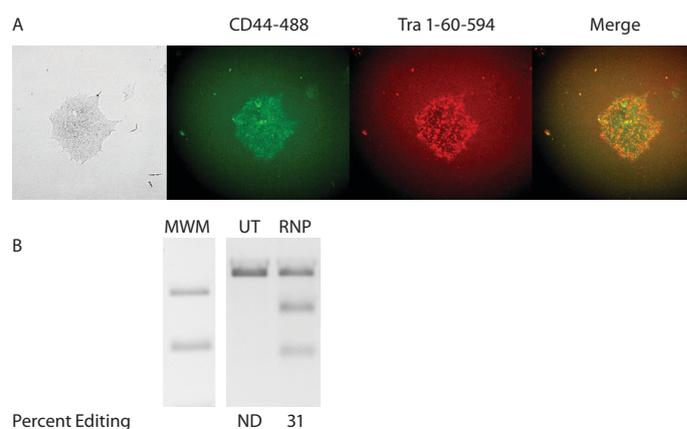


Figure 1: Working with iPS cells – Checking for differentiation and gene editing
A. Determination of iPS cell differentiation using live cell imaging of CD44 Alexa Fluor 488 (differentiated) and Tra-1-60 Alexa Fluor 594 (undifferentiated) fluorescently labeled primary antibodies to each cell surface marker. **B.** DNA mismatch detection assay of *PP1B*-targeting crRNA.

Knockout of *POU5F1* using Edit-R Cas9 nuclease protein causes iPS cells to differentiate

POU5F1 has previously been shown to be essential for the maintenance of iPS cell pluripotency⁽⁷⁾. That led us to propose the following question: If crRNAs are designed to program Cas9 to knockout *POU5F1*, will iPS cell pluripotency be affected, and would iPS cells differentiate? To investigate this, five synthetic crRNAs were designed to target the coding region of *POU5F1* with the proprietary Edit-R guide RNA design algorithm. Using previously described nucleofection conditions we individually delivered each of the five different crRNAs into undifferentiated iPS cells with Cas9 protein as part of an RNP complex. Seventy-two hours after transfection, live cell differentiation markers were used to determine differentiation caused by Cas9 targeting *POU5F1* (Figure 2A). Sub-populations of iPS cells displaying increased differentiation staining were visualized, while the untransfected and the non-targeting crRNA controls showed very low amounts of differentiation (Figure 2A). After live cell imaging, each individual crRNA cell population was lysed and a subsequent mismatch detection assay (T7EI) was performed, which confirmed detectable levels of editing (indels) at *POU5F1* (Figure 2B).

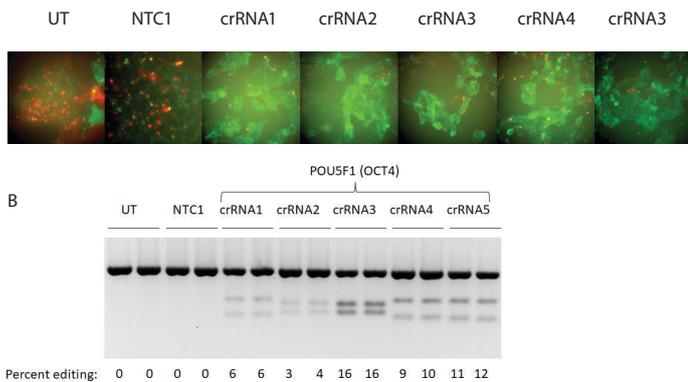


Figure 2: Inducing iPS cell differentiation with synthetic crRNAs
A. CD44 Alexa Fluor 488 and Tra-1-60 Alexa Fluor 594 stained live iPS cells either untransfected or transfected with NTC1 crRNA of five different crRNAs targeting *POU5F1*. **B.** DNA mismatch detection assay of NTC1 or *POU5F1*-targeting crRNA.

Activating gene expression with CRISPRmod CRISPRa dCas9-VPR and synthetic guide RNA

CRISPR activation (CRISPRa) is a powerful gene modulation tool that uses a nuclease-deactivated Cas9 (dCas9) fused to the chimeric gene activation domain VPR (VP64, P65 and Rta) to increase the transcription of an endogenous gene target⁽⁸⁾. CRISPRmod CRISPRa dCas9-VPR was used in conjunction with CRISPRmod CRISPRa synthetic crRNAs targeting *POU5F1*, *TTN*, *ASCL1*, or *EGFR*. A plasmid driving the expression of dCas9-VPR was co-delivered with each gene specific synthetic crRNA:tracrRNA. Seventy-two hours after nucleofection, RT-qPCR was used to determine the levels of gene activation (Figure 3A). Target gene transcription levels of three gene targets were detected at increased levels when compared to untransfected controls (Figure 3A). We did not see further activation of *POU5F1* in this experiment because it is known that *POU5F1* is highly expressed and essential to maintain pluripotency in iPS cells⁽⁷⁾. Of the three gene targets that were successfully activated, *ASCL1* transcript levels were increased to over 100-fold compared to untransfected cells. *ASCL1* is a member of the basic helix-loop-helix (BHLH) family of transcription factors and this protein has been described as an oncogene and suggested to be essential for the survival of lung cancers⁽⁹⁾. We wanted to check if activation of *ASCL1* would lead to downstream effects and activation of previously described gene targets⁽⁹⁾. Interestingly, we probed the gene expression levels of two previously characterized downstream *ASCL1* interaction proteins⁽⁹⁾, *DLL1* and *DLL3*, and saw that the *DLL1* transcript was increased three-fold and the *DLL3* transcript levels increased by approximately 30-fold over untransfected controls (Figure 3B). This combination of CRISPRa and iPS cells represents an interesting experimental system as one could begin to test questions associated with increased gene expression levels on the differentiation of specific cell types.

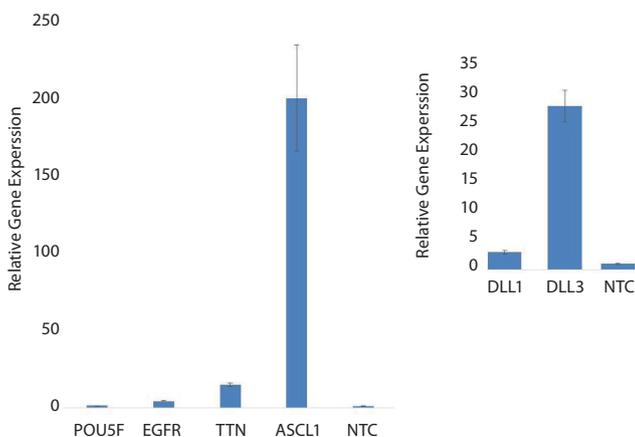


Figure 3: Performing CRISPRa in iPS cells

A. dCas9-VPR and a synthetic crRNA targeting either *POU5F1*, *TTN*, *ASCL1*, *EGFR*, or a non-targeting control (NTC) were delivered to iPS cells with nucleofection. Gene activation of *POU5F1*, *TTN*, *ASCL1*, *EGFR*, or NTC was assessed at seventy-two hours post nucleofection by RT-qPCR and is shown as fold transcriptional activation compared to the NTC. **B.** Relative expression of *DLL1*, *DLL3*, or NTC upon CRISPRa transcriptional activation of *ASCL1* analyzed by RT-qPCR and normalized to NTC samples.

Knock-in of EGFP

Using CRISPR-Cas9 to create knockout cell lines is an important tool for teasing apart the function of a specific gene target. Conversely, there are circumstances when one would want to repair a genetic defect, create a reporter cell line, or tag an endogenous protein. Here iPS cells were nucleofected with Cas9 protein, *SEC61B* crRNA, tracrRNA, and an EGFP repair plasmid. The repair plasmid was designed to insert a single EGFP tag into the N-terminus of *SEC61B* using 1,000 bp homology arms. Transfected cells were grown for an additional 72 hours before imaging medium was applied, and cells were visualized on a fluorescent microscope for proper localization (Figure 4). After imaging, cells were disassociated and the population of GFP positive cells was determined by flow cytometry. The HDR-mediated knock-in of EGFP was observed in 2.6% of the cell population as measured by FACS.

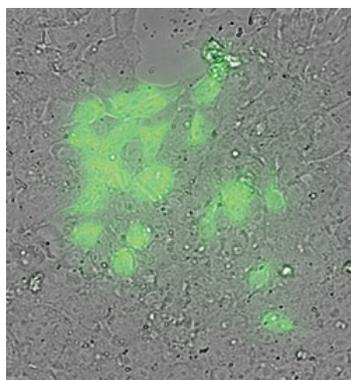


Figure 4: Creating an EGFP reporter in iPS cells using HDR
 EGFP fluorescence in iPS cells of HDR tagged EGFP-SEC61B

Discussion

Here we have demonstrated the effectiveness of Edit-R CRISPR-Cas9 and CRISPRmod CRISPRa reagents for gene editing and gene modulation in a human iPS cell line. The use of the synthetic two-part crRNA:tracrRNA system in iPS cells provides rapid reagent generation and compatibility with different CRISPR-Cas9 systems. CRISPR-Cas9 mediated knockout of genes required for the maintenance of pluripotency, such as *POU5F1*, can be targeted to drive differentiation into different cell types, or in a screen to identify other gene targets important in the maintenance of pluripotency. Researchers can also use CRISPR-Cas9 and iPS cells to create matched, isogenic cell lines whereby an unmodified iPS cell population can be compared to a CRISPR-Cas9 targeted iPS cell population to study the impact of a specific genotype on a cellular phenotype.

We were able to show that CRISPRa reagents showed an increase in gene expression of a lineage specific oncogene (*ASCL1*) and subsequent downstream transcriptional activation of *ASCL1* gene target, *DLL3*. CRISPRa provides the ability to begin to ask questions related to elevated expression levels of specific and downstream gene targets, and the compatibility of CRISPRa with synthetic crRNA enables both a pooled and arrayed approach to test these questions. One such question to take this experiment further would be to compare gene expression profiles, protein interaction partners, etc. in elevated *ASCL1* iPS cells where *ASCL1* is not oncogenic, to iPS cells differentiated into pulmonary neuroendocrine cells where *ASCL1* is oncogenic.

Finally, we were able to create a reporter iPS cell line using Cas9 RNP and an EGFP repair plasmid to tag the N-terminus of SEC61B. The methods presented in the creation of the reporter cell line can be adopted to create other precisely tagged, or repaired gene targets such as the creation of disease causing SNPs, or the creation of differentiated cell reporters.

This application note demonstrates the utility of synthetic crRNA, tracrRNA, and Cas9 protein as a convenient source of CRISPR reagents for effective genome editing in human iPS cells.

Materials and methods

Tissue culture: iPS cells (Thermo, Cat#A18945) were maintained according to the manufacturer's recommendations in Essential 8™ medium (Thermo, Cat #A1517001), on vitronectin coated culture plates (Thermo, Cat# A14700) in serum-free conditions. Cells were passaged using ReLeSR, an enzyme-free dissociation reagent (Stem Cell technologies, Cat# 05872) according to manufacturer's recommendations. During passaging, iPS cells were supplemented with RevitaCell Supplement (Thermo, Cat# A26445). Essential 8 medium was replaced every twenty-four hours during culture.

Electroporation: At the time of electroporation, 2×10^5 (96-well Shuttle Nucleofection System) or 8×10^5 iPS (Lonza 2b Nucleofection System) cells were collected per reaction and centrifuged at $500 \times g$ for 2 minutes. Cell pellets were washed with DPBS (GE Healthcare Hyclone, Cat #SH30264.01) and re-centrifuged at $500 \times g$ for an additional 2 minutes. Cells were resuspended in 100 μ L Buffer P3 (Lonza, Cat # PBP3-00675). For the 96-well shuttle system, cells were then mixed with 30 pmol Edit-R Cas9 Nuclease protein NLS (Dharmacon, Cat #CAS11729; 40 μ M), and 60 pmol tracrRNA (Dharmacon, Cat #U-002000-120) with 60 pmol predesign Edit-R PPIB crRNA (Dharmacon, Cat #CM-004606-02-0020) or 60 pmol Edit-R crRNA Nontargeting Control #1 (Dharmacon, Cat # U-007501-01-20). For the 2b nucleofection system, cells were then mixed with 500 ng dCas9-VPR plasmid and 500 ng custom sgRNA plasmid. For HDR experiments 500 ng repair template was used. Nucleofections were carried out, as indicated, with either the Lonza Nucleofector™ 96-well Shuttle with the P3 Primary Cell Solution (Lonza, Cat# PBP3-02250) or the Lonza Nucleofector™ 2b with the Human Stem Cell Nucleofector Solution 2 (Lonza, Cat# VPH-5002) according to the manufacturer's protocol.

DNA mismatch detection assay: Cells (1×10^5 per sample) were lysed in Phusion™ HF buffer (Thermo Scientific, Cat #F-518) with 5 μ L each of Proteinase K (Thermo Scientific, Cat #EO0492) and RNase A (Thermo Scientific, Cat #EN0531), and incubated for 1 hour at 56 °C then heat inactivated at 98 °C for 10 minutes. Fifty microliter PCR reactions were carried out using 0.5 μ L Phusion Hot Start II DNA Polymerase (Thermo Scientific, Cat #F-549S), 5X Phusion HF buffer (Thermo Scientific, Cat #F-518), 200 μ M each dNTP (Thermo Scientific, Cat #F-549S), 0.5 μ M forward and reverse primers (PPIB: Forward 5'-ACCGTGATTTTACCACGAAAT-3', Reverse 5'-AAACATTCGTAGGTCAAAATACA-3'; POU5F1: Forward 5'-AGAAGGGCAAGCGATCAAGC-3', Reverse 5'-TGAGCATTGGATATCCCATCC-3') and 5 μ L direct cell lysis template. Touchdown PCR and an annealing program with the following thermal cycling steps were run for each sample: Denature/enzyme activation at 98 °C for 3 minutes followed by 10 cycles of 98 °C for 10 seconds, 72 °C for 15 seconds -1 °C/cycle, and 72 °C for 30 seconds then 25 cycles of 98 °C for 10 seconds, 62 °C for 15 seconds and 72 °C for 30 seconds and final extension at 72 °C for 10 minutes. Samples were heated to 95 °C for 10 minutes and slowly cooled to re-anneal. Then, 10 μ L of annealed PCR

products were combined with 5 units of T7E1 enzyme and NEBuffer 2 (New England Biolabs, Cat #M0302L) and incubated at 37 °C for 25 minutes. Three microliters of 6X Orange Loading Dye (Thermo Scientific, Cat #R0631) was added to the T7E1 reactions and the entire volume was loaded and ran on a 2% agarose gel. For more details see the Edit-R Synthetic crRNA Positive Controls Protocol. The level of editing was calculated using densitometry (% gene editing) in Image J and gene editing was estimated with the equation $(1-\sqrt{(1-(a+b)/(a+b+c))}) \times 100$.

Gene expression analysis (RT-qPCR): Total RNA was isolated using an SV 96 Total RNA Isolation System (Promega, Cat #Z3500) per manufacturer's instructions. cDNA was synthesized using a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Cat#K1672). Briefly, 1 μ L of 10X dsDNase Buffer, 1 μ L of dsDNase, and 8 μ L of total isolated RNA were combined and incubated at 37 °C for 2 minutes. Next, 4 μ L of 5X Reaction Mix, 2 μ L of Maxima Enzyme Mix, and 4 μ L of nuclease-free water were added to each reaction. Each reaction was incubated at 25 °C for 10 minutes followed by 30 minutes at 50 °C and terminated by incubating at 85 °C for 5 minutes. Relative gene expression was determined using TaqMan Gene Expression Assays. For each assay, 7.5 μ L of TaqMan Master Mix was combined with 2 μ L of undiluted cDNA, 0.75 μ L of respective TaqMan Probe, and 4.75 μ L of nuclease-free water. Samples were mixed and 10 μ L of each reaction was added to individual wells on a 384-well plate and loaded onto a Roche LightCycler 480 II. The following thermo cycling steps were performed on each sample: Enzyme activation at 95 °C for 10 minutes followed by 40 cycles of denaturing at 95 °C for 15 seconds and annealing at 60 °C for 1 minute. Each sample was performed in technical triplicate. The relative expression of each gene was calculated with the $\Delta\Delta Cq$ method using GAPDH as the housekeeping gene and normalized to a non-targeting control.

Fluorescence microscopy: CD44 Alexa Fluor 488 (Thermo, Cat #A25528) and Tra-1-60 Alexa Fluor 594 (Thermo, Cat #A24882) were used according to manufacturer's recommendations. CD44 Alexa Fluor 488 and Tra-1-60 Alexa Fluor 594 were added to the iPS cell culture media, after a 30-minute incubation at 37 °C, culture media was washed twice with FluoroBrite DMEM (Thermo, Cat #A1896701) and immediately imaged.

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