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Efficient footprint-free gene editing of hiPS cells using CRISPR/Cas9

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Abstract

The combination of two powerful technologies, human induced pluripotent stem cells (hiPSCs) and precise, footprint-free editing using CRISPR/Cas9, allows for a new level of sophistication in the development of disease models. The ability to create hiPS cell lines from donors with disease-specific mutations and to edit mutations into specific genomic backgrounds will enable discoveries with a new level of granularity. Despite progress in improving nuclease specificity and reducing off-target activity with precise tools like CRISPR/Cas9, a major challenge for successful gene editing in hiPSCs is the lack of culture systems that allow researchers to isolate single hiPSCs with the desired mutations and to generate stable, healthy, clonal lines from edited cells. Traditionally, hiPS cells are grown and passaged as colonies. In order to obtain single cells for cloning, the colonies must first be dissociated into a single-cell suspension, which often results in cell death or premature differentiation. Furthermore, gene editing protocols often subject stem cells to harsh conditions that compromise their health and survival. Using the Cellartis® DEF-CS™ 500 Culture System, we can culture hiPS cells in a monolayer with a very high rate of single-cell survival and clone expansion. We used this culture system to develop a complete workflow, starting with CRISPR/Cas9-mediated editing, using Cas9/sgRNA ribonucleoprotein (RNP) complexes delivered into hiPS cells via either electroporation or cell-derived nanoparticles called gesicles, followed by successful single-cell cloning of edited hiPS cells. We chose non-DNA-based delivery methods to guarantee footprint-free editing of the hiPS cells. We achieved endogenous gene knockout efficiencies of up to 65% for the membrane protein CD81 in a hiPS cell population. We also achieved efficient, accurate knockin using electroporation with long ssDNA donor fragments. We demonstrated that edited hiPS clones obtained with this workflow were still pluripotent and had a normal karyotype, even after further expansion. The data show this workflow using footprint-free editing via efficient delivery of Cas9/sgRNA RNP complexes and single-cell cloning of hiPS cells in modified media, results in a high number of edited and expandable hiPS cell clones that maintain the hallmarks of pluripotency.

1 DEF-CS system improves single-cell cloning efficiency of edited cells

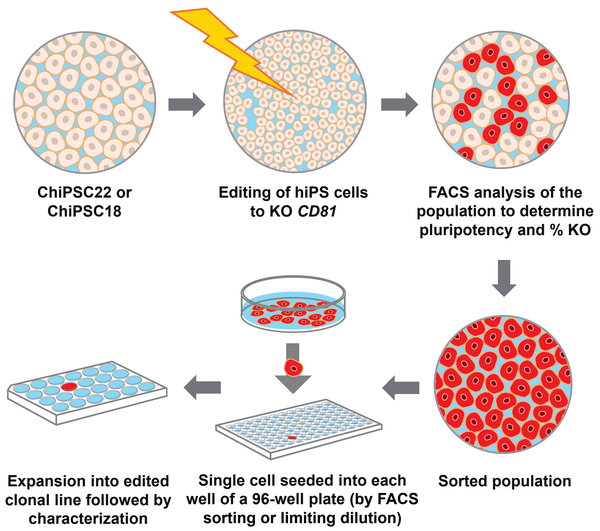

Figure 1. Workflow for targeted knockout of an endogenous gene *(CD81)* in hiPSC cells using the DEF-CS system. After adapting hiPS cells to the DEF-CS system, cells expand into a karyotypically stable, pluripotent monolayer. To edit hiPS cells, a complex of recombinant Cas9 and sgRNA, forming a ribonucleoprotein (RNP) complex, is delivered into the cells using electroporation. Knockout of *CD81* in the cell population is monitored via FACS analysis to determine the percentage of edited cells in the population. FACS analysis is also used to monitor maintenance of pluripotency of edited cells. Since the target gene permits selection of the edited population by flow cytometry (negative for CD81, positive for pluripotency marker SSEA-4), the target population can be sorted followed by single-cell cloning to create a edited clonal cell lines.

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2 DEF-CS system maintains hiPS-cell pluripotency after *CD81* KO

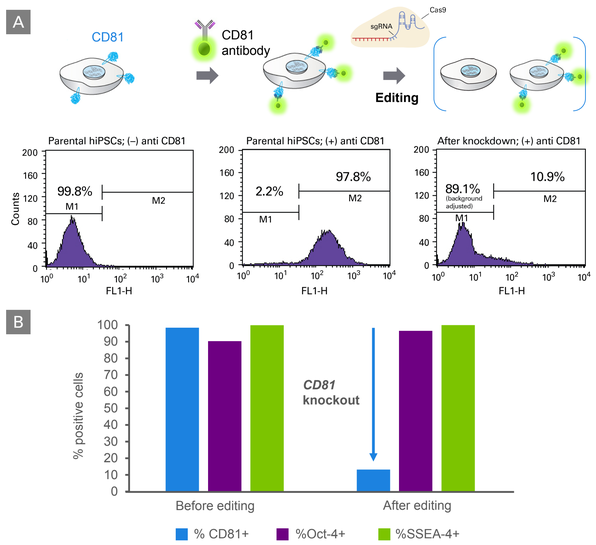

Figure 2. The DEF-CS system maintains hiPS cell pluripotency after *CD81* KO. Panel A. ChiPSC18 cells (Cat. # Y00305) were edited via electroporation of recombinant Cas9 (Cat. # 632640) and *in vitro*-transcribed sgRNA (generated using Cat. # 632639) targeting the *CD81* gene locus. The expression of CD81, as well as the pluripotency of the cells after the editing experiment, was quantified via FACS using fluorescently labeled antibodies against CD81, Oct-4, and SSEA-4. Almost 90% of cells had *CD81* knocked out. **Panel B.** More than 90% of edited cells were positive for Oct-4 and SSEA-4 expression.

3 DEF-CS system allows for single-cell cloning of edited hiPS cells

Cell line	Isolation method	Single clones	Double clones	Total clones (proportion)	Total clones (%)
ChiPSC22	FACS	8	0	8/96	8.5%
ChiPSC22	Limiting dilution	39	15	54/55	98%
ChiPSC18	FACS	52	0	52/96	54%
ChiPSC18	Limiting dilution	46	12	58/55	105%

Figure 3. The DEF-CS culture system allows for high single-cell cloning efficiency of edited hiPSCs. Edited cells were seeded in a 96-well plate via FACS or limiting dilution, using a final dilution equivalent to 0.5 cells/well. The number of wells with expanding, edited clonal cell lines were counted one week after seeding.

4 DEF-CS system maintains the pluripotency of the edited and isolated hiPS cell clones

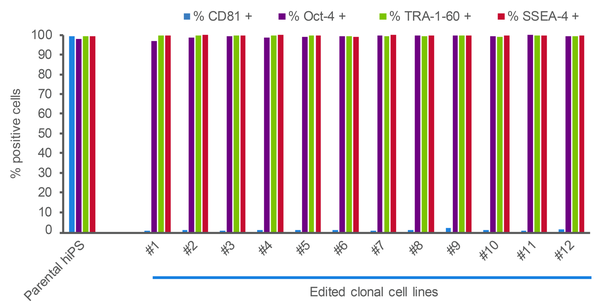

Figure 4. Analysis of pluripotency of edited and isolated hiPS cell clones. Individual, edited (*CD81* knockout) hiPS cells, were expanded into clonal lines and analyzed for expression of CD81 and three pluripotency markers via FACS using antibodies against CD81, Oct-4, TRA-1-60, and SSEA-4. The parental hiPS cell line ChiPSC18 was used as a positive control. As expected, all edited clones exhibited the loss of CD81 expression. Pluripotency was maintained in all edited clonal lines, as evidenced by the persistent expression of the three pluripotency markers.

5 DEF-CS system maintains the karyotype of the edited and isolated hiPS cell clones

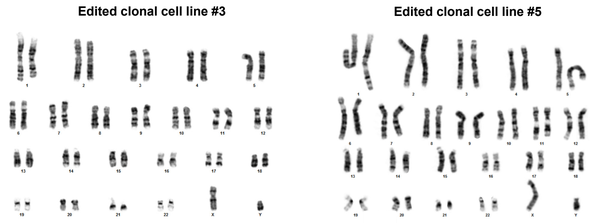

Figure 5. Analysis of the karyotype of edited and isolated hiPS cell clones. The karyotype of the five edited clonal cell lines was analyzed. All clonal lines showed the expected 46, XY karyotype of the original ChiPSC18 cell line. Clonal cell lines #3 and #5 are shown as examples.

6 Homology-directed knockin of point mutations in hiPSCs

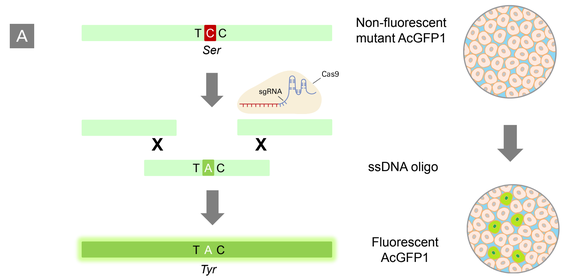

Figure 6. Correction of the sequence encoding for fluorescent protein *(AcGFP1)* in hiPS cells by homology-directed repair (HDR) using CRISPR/Cas9. Panel A. ChiPSC18 stable cell line expressing a mutant/non-fluorescent AcGFP1 was electroporated with a Cas9/sgRNA RNP complex and a single-stranded DNA oligo (ssDNA Oligo) as the donor repair template. This oligo was bearing a 5' and 3' specific homology sequence flanking the intended sequence change, a point mutation from C to A, that would generate a fluorescent variant of AcGFP1 after a successful, in-frame HDR. **Panel B.** The efficiency of *AcGFP1* gene repair via HDR was determined by flow cytometry. A maximum repair efficiency of 28% was obtained by using 1 µg ssDNA donor in the electroporation. **Panel C.** A decrease in the percentage of AcGFP1 fluorescent-positive cells could be detected by flow cytometry if the amount of ssDNA repair donor oligo electroporated was lower or higher than 1 µg.

7 Precise and efficient insertion of a fluorescent protein in the *AAVS1* site using a long single-stranded oligo as the donor template

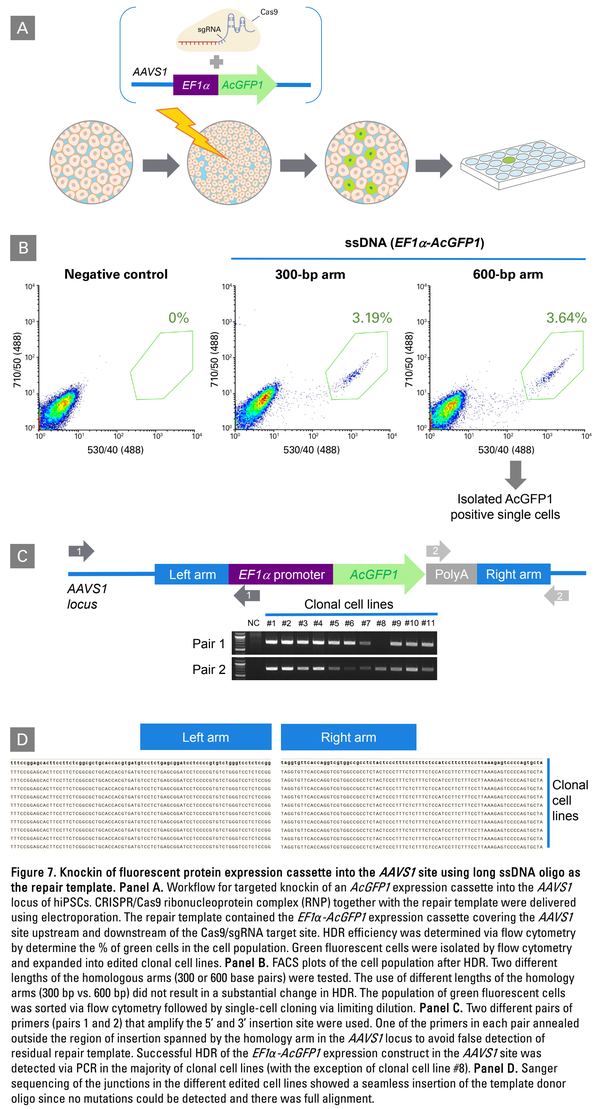

Figure 7. Knockin of fluorescent protein expression cassette into the *AAVS1* site using long ssDNA oligo as the repair template. Panel A. Workflow for targeted knockin of an *AcGFP1* expression cassette into the *AAVS1* locus of hiPSCs. CRISPR/Cas9 ribonucleoprotein complex (RNP) together with the repair template were delivered using electroporation. The repair template contained the *EF1α-AcGFP1* expression cassette covering the *AAVS1* site upstream and downstream of the Cas9/sgRNA target site. HDR efficiency was determined via flow cytometry by determine the % of green cells in the cell population. Green fluorescent cells were isolated by flow cytometry and expanded into edited clonal cell lines. **Panel B.** FACS plots of the cell population after HDR. Two different lengths of the homologous arms (300 or 600 base pairs) were tested. The use of different lengths of the homology arms (300 bp vs. 600 bp) did not result in a substantial change in HDR. The population of green fluorescent cells was sorted via flow cytometry followed by single-cell cloning via limiting dilution. **Panel C.** Two different pairs of primers (pairs 1 and 2) that amplify the 5' and 3' insertion site were used. One of the primers in each pair annealed outside the region of insertion spanned by the homology arm in the *AAVS1* locus to avoid false detection of residual repair template. Successful HDR of the *EF1α-AcGFP1* expression construct in the *AAVS1* site was detected via PCR in the majority of clonal cell lines (with the exception of clonal cell line #8). **Panel D.** Sanger sequencing of the junctions in the different edited cell lines showed a seamless insertion of the template donor oligo since no mutations could be detected and there was full alignment.

Conclusions

- The combination of successful genome editing and the establishment of edited, pluripotent hiPSC clones via the DEF-CS culture system is essential to create disease model systems.
- Editing of hiPS cells can be accomplished either via electroporation (using rCas9 and *in vitro*-transcribed sgRNA) or via gesicle-based delivery of the Cas9/sgRNA RNP complex.
- The DEF-CS culture system allows the establishment of single-cell clones from edited hiPS cells with very high efficiency.
- Colonies obtained from these single, edited cells maintain pluripotency and karyoptype when grown in the DEF-CS culture system.