Footprint-free gene editing using CRISPR/Cas9 and single-cell cloning of edited human iPS cells

Christopher Sontag, Montse Morell, Tatiana Garachtchenko, Hiro Matsumoto, Michael Haugwitz, and Andrew Farmer

Takara Bio USA, Inc., Mountain View, CA, USA; Corresponding author: michael_haugwitz@takarabio.com

Abstract

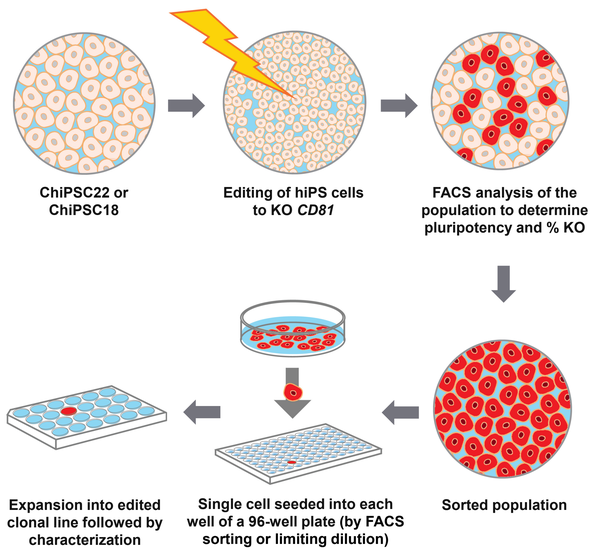
The combination of two powerful technologies (human induced pluripotent stem [hiPS] cells and precise, footprint-free editing using CRISPR/Cas9) allows for a new level of sophistication in cell biology research and disease model development. The ability to create hiPS cell lines from different donors and to determine the effects of specific mutations created via gene editing within the donor-specific genetic background will enable discoveries with a new level of granularity.

However, while the introduction of CRISPR/Cas9 technology has made gene editing easier to achieve (even in hiPS cells), obtaining single-cell clones of edited hiPS cells has been a major bottleneck. Traditionally, hiPS cells are grown and passaged as colonies. In order to obtain single cells for cloning purposes, the colonies must first be dissociated into a single-cell suspension, which often results in cell death or premature differentiation. In contrast to traditional methods, the Cellartis® DEF-CS™ 500 Culture System allows culturing of hiPS cells in a monolayer and permits single-cell isolation. The specific compositions of the DEF-CS culture medium and coating reagent allow for a very high rate of single-cell survival and clone expansion.

We applied 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 (KO) efficiencies of up to 65% for the membrane protein CD81 in a hiPS cell population. Individual, edited hiPS cells were seeded into a 96-well plate either by limiting dilution or fluorescence activated cell sorting. Notably, the use of limiting dilution resulted in a very high recovery rate of single hiPS cells which went on to form clonal colonies. We also demonstrated that edited hiPS clones obtained with the described workflow were still pluripotent, 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 using a modified DEF-CS culture protocol, results in a high number of edited and expandable hiPS clones that maintain the hallmarks of pluripotency.

Workflow for targeted knockout of an endogenous gene in hiPS cells using the DEF-CS system



1 *AcGFP1* KO hiPS cells remain pluripotent in the DEF-CS system

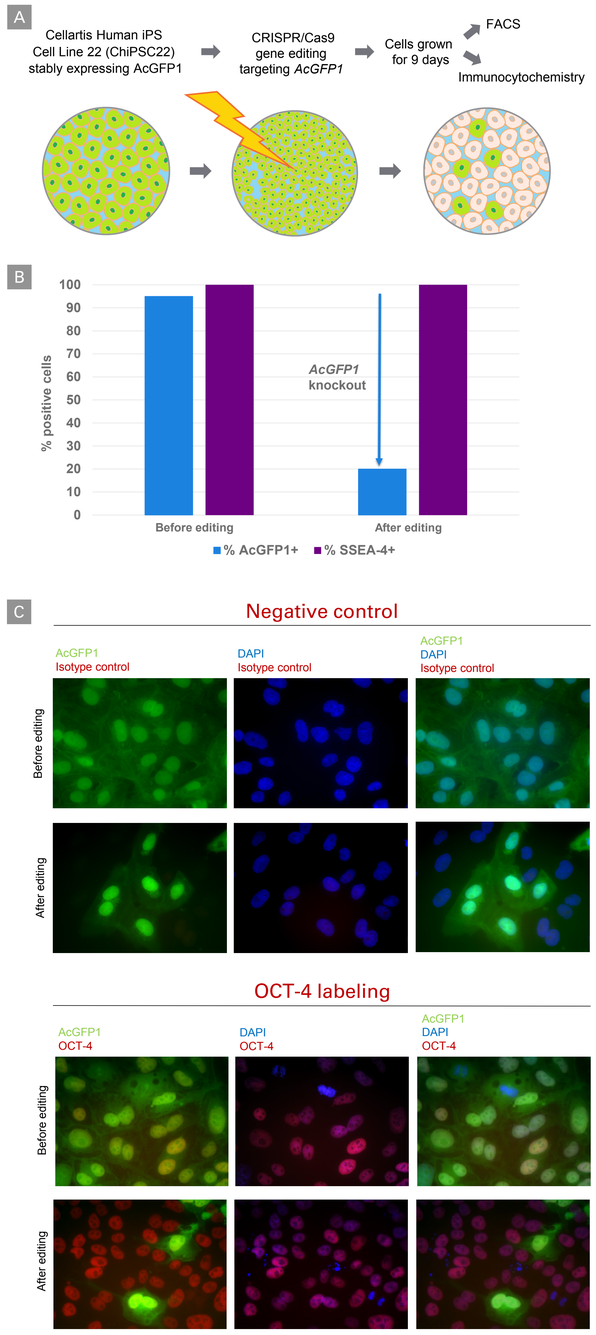


Figure 1. ***AcGFP1*** **KO hiPS cells remain pluripotent in the DEF-CS system. Panel A.** Gene editing to knockout *AcGFP1* was performed on Cellartis Human iPS Cell Line 22 (ChiPSC22, Cat. # Y00325) stably expressing AcGFP1 using the Guide-it™ CRISPR/Cas9 Gesicle Production System (Cat.# 632613). Cells were cultured under non-differentiating conditions using the Cellartis DEF-CS 500 Culture System (Cat. # Y30010). After editing, cells were analyzed for AcGFP1 expression and pluripotency via FACS **(Panel B)** and immunocytochemistry **(Panel C)**. As a negative control, unedited parental cells were grown in parallel for the same amount of time. The quantification of AcGFP1 expression in the edited cells revealed an 80% knockout. Further, pluripotency of parental and edited cell populations was maintained, with nearly 100% of cells expressing SSEA-4 (assessed via FACS in Panel B) and OCT-4 (assessed via immunohistochemistry in Panel C).

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

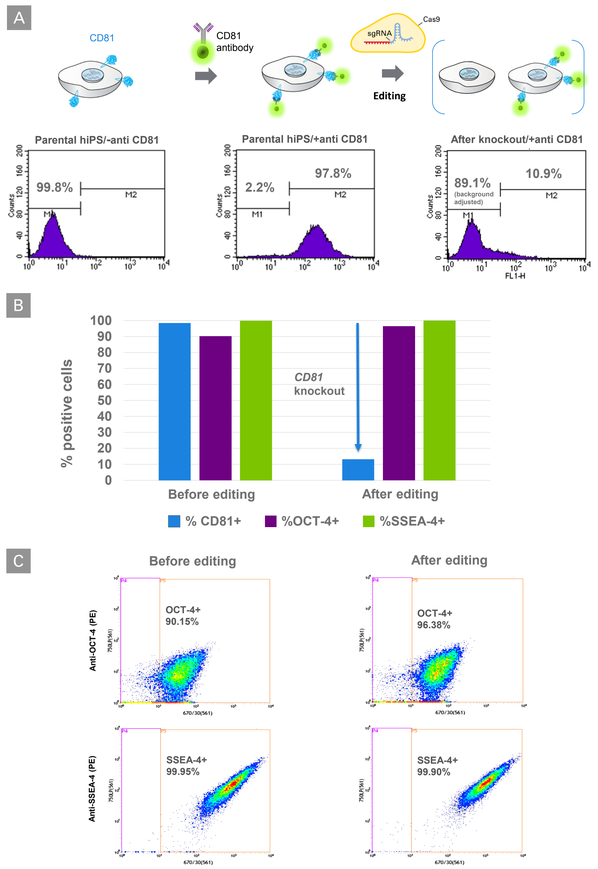


Figure 2. The DEF-CS system maintains hiPS cell pluripotency after *CD81* KO. Panel A. ChiPSC18 (Cat. # Y00305) cells were edited via electroporation of recombinant Cas9 and *in vitro*-transcribed sgRNA targeting the *CD81* gene locus. The expression of CD81, as well as the pluripotency of the cells 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 maintained pluripotency (>90% of the cells were positive for OCT-4 and SSEA-4 expression). **Panel C.** Representative FACS plots show the maintained expression of the pluripotency markers OCT-4 and SSEA-4 in cells before as well as after editing.

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

A

ChiPSC cell line	Isolation method	Single clones	Double clones	Total clones (proportion)	Total clones (%)
ChiPSC18	FACS	4	0	4/96	4.2%
ChiPSC18	Limiting dilution	33	7	40/50	80%

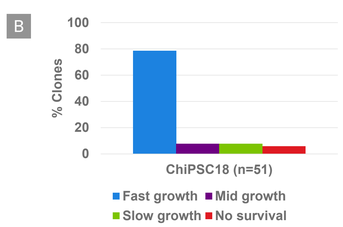


Figure 3. The DEF-CS culture system allows for high single-cell cloning efficiency of edited hiPSCs. Panel A. Edited cells were seeded in a 96-well plate via FACS or limiting dilution, using a final dilution equivalent to 0.5 cells/well. The number of growing clonal cell lines were counted one week after seeding. **Panel B.** Two weeks post-seeding, the growth rates of 51 emerging colonies were qualitatively characterized. Almost 80% of the analyzed, edited clones exhibited fast and robust growth in the 96-well plate using the DEF-CS culture system.

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

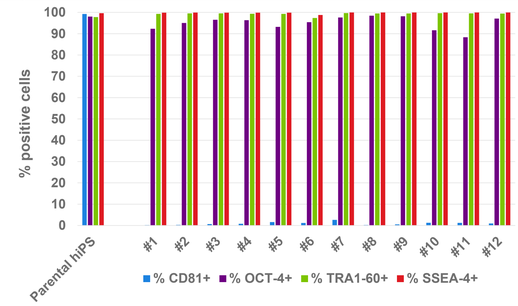


Figure 4. The DEF-CS system maintains pluripotency of the edited and isolated hiPS cell clones. Individual, edited (*CD81* knockout) hiPS cells, isolated via limiting dilution, were expanded into clonal lines and analyzed for expression of CD81 and pluripotency markers via FACS using antibodies against CD81, OCT-4, TRA1-60, 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. However, pluripotency was maintained in all edited clonal lines, as evidenced by high expression of the three pluripotency markers.

5 Genomic characterization of clones with incomplete KO of *CD81*

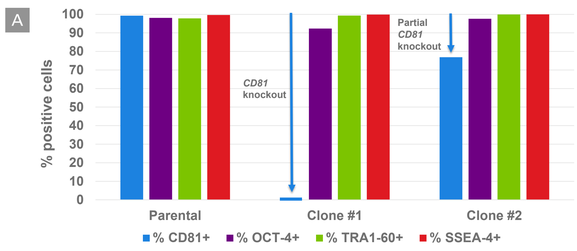


Figure 5. Monoallelic and biallelic editing of the *CD81* genome locus in different hiPS clones. Panel A. Some hiPS clones obtained after targeting the genomic *CD81* locus via CRISPR/Cas9 (electroporation) showed a complete loss of CD81 expression (Clone #1). However, other clones only exhibited a reduction but not a complete loss of CD81 expression (Clone #2). Both clones exhibited expression of all three pluripotency markers (OCT-4, TRA1-60, and SSEA-4), comparable to the parental, non-edited hiPS cell line. **Panel B.** The genomic *CD81* region of those two edited hiPS clones as well as the parental hiPS cell line were PCR amplified and sequenced to obtain detailed sequence information regarding the two *CD81* alleles of the respective clones. The results showed that Clone #1 is a heterozygous, biallelic knockout for *CD81*, which explains the complete lack of CD81 expression in this clone. However, Clone #2 is a monoallelic mutant for the *CD81* locus, containing one wild-type allele and one mutant allele. This indicates that the reduced expression level is the result of expression of only one allele, compared to the expression of CD81 from both alleles, in the parental hiPS cell line.

Conclusions

- The combination of successful genome editing and the establishment of edited, pluripotent hiPS 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 when grown in the DEF-CS culture system.
- Gene editing using CRISPR/Cas9 can result in either mono- or biallelic editing in hiPS cells.

Scan to download your copy of this poster or visit
takarabio.com/ASCB-2016-Cas9-hiPS-Poster

800.662.2566
Visit us at takarabio.com/stemcells

Clontech TaKaRa cellartis

01.17 US (633842)