Techniques for CRISPR/Cas9-Facilitated Genome Editing and Single-Cell Cloning of hPSCs

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INTRODUCTION

Induced pluripotent stem cells (iPSCs) are versatile *in vitro* model systems for studying human disease mechanisms directly in patient-derived cells. CRISPR/Cas9-faciliated genome editing has enabled efficient genetic manipulations for many mammalian cell types. Together, these two technologies have synergistic potential to create a vast number of new discovery tools for biomedical research. Yet genome editing in human iPSCs still poses many challenges, such as ensuring consistent delivery of CRISPR/Cas9 reagents into iPSCs, optimizing screening workflows to conserve reagents, and maintaining pluripotency and genomic stability in edited clonal cell lines. We found that iPSCs adapted to single-cell passaging transfect more efficiently and demonstrate high rates of indel formation using sgRNA-Cas9 ribonucleoprotein (RNP) complexes. Edited clones retained a normal karyotype after screening. We used a combination of various commercially available reagents and analyzed genomic DNA sequencing results using a web-based bioinformatics software. Finally, we saw greater viability and continued growth of iPSCs that were sorted and cloned as single cells using a microfluidics chip-based cell sorter. In sum, our results show that efficient genome editing in human iPSCs is possible using currently available reagents and techniques.

Tuberous sclerosis complex (TSC) is a rare genetic disorder caused by mutations in either TSC1 (hamartin) or TSC2 (tuberin), two tumor suppressor genes that regulate the mTOR pathway. **We selected the TSC1 gene as a KO target and here we demonstrate high efficiency of biallelic targeting using CRISPR/Cas9-based genome editing.**

MATERIALS & METHODS

Cell lines and cell culture reagents

Human iPSCs used: M22c5 (**BCMi001-A**) and M10c5 iPSCs. Feeder-free media used: mTeSR1 (STEMCELL Technologies), StemFlex (Thermo Fisher Scientific). Substrate used: hESC-qualified Matrigel (Corning). Cell-cluster passaging was performed using ReLeSR (STEMCELL Technologies). Single-cell dissociation was performed using Accutase and by supplementing with 5μM ROCK inhibitor to the growth medium for the first 24 hours.

4D-Nucleofection of CRISPR/Cas9 reagents

Ribonucleoproteins (RNPs), consisting of a modified sgRNA (Synthego) targeting a common exon of TSC1 complexed to different Cas9 recombinant proteins (Synthego, Thermo Fisher, Millipore-Sigma, or Aldevron), were delivered into M10c5 iPSCs by nucleofection. 4D-Nucleofection was performed using Lonza's Primary Cell P3 solution and Program CA-137.

Sanger sequencing and bioinformatics analysis

100ng of genomic DNA was used to amplify regions of interest. Spin columni-purified PCR products were submitted for Sanger sequencing (Eurofins). Efficiency of indel formation by non-homologous end-joining (NHEJ) was analyzed using the Inference of CRISPR Edits (ICE) bioinformatics software (Synthego).

Single-cell sorting

The WOLF® Cell Sorter and N1 Single-Cell Dispenser system (NanoCellect Biomedical) was used to sort and deposit iPSCs as single cells into Matrigel-coated 96-well plates. Single cell-derived iPSC clones were grown in StemFlex (Thermo Fisher Scientific), supplemented with 10μM ROCK inhibitor for the first 24 hours.

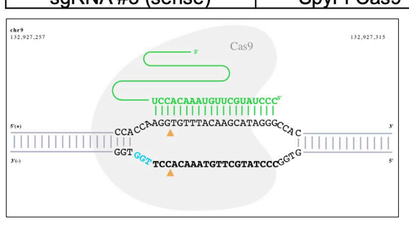
Simple Western blotting

Size-based protein separation and chemiluminescence detection was performed on the ProteinSimple Wes instrument using 12-230 kDa Wes Separation Module (#SM-W003). Briefly, total protein was extracted from iPSCs cultured in triplicate and 0.6μg of protein was separated per capillary. Antibodies used were: mouse anti-human TSC1 (R&D Systems, #488915, 1:50 dilution), mouse anti-α-tubulin (Abcam, #ab7291, 1:1000 dilution), rabbit anti-phospho-p70 S6 Kinase antibody (R&D Systems, #AF8965, 1:50 dilution), and goat anti-mouse or anti-rabbit HRP-conjugated secondary antibodies (ProteinSimple #042-205 or 206). Optimal sample and antibody dilutions and specificity of primary antibodies were determined prior to the final experiment. Data were analyzed and prepared by the Compass software for Simple Western instruments version 4.0.0 and GraphPad Prism version 8.1.2.

FIGURE 1

Optimization of CRISPR/Cas9 reagents for targeting TSC1. Three TSC1-specific crRNAs were designed using CRISPR Design Tool (Synthego) and target sequence #3 was selected based on the ICE KO-Scores. The same crRNA sequence was synthesized as a sgRNA and 4 different brands of Cas9 were compared using the KO-Score as readout. Three TSC1 frameshift mutant clones, with precise 11-nt deletions identified by genomic PCR and Sanger sequencing, display reduced TSC1 mRNA levels.

Name	Cas9	ICE	R^2	KO-Score
crRNA #1 (anti-sense)	2NLS Cas9	6	0.99	3
crRNA #2 (anti-sense)	2NLS Cas9	9	0.99	7
crRNA #3 (sense)	2NLS Cas9	24	0.99	23
sgRNA #3 (sense)	2NLS Cas9	22	0.99	21
sgRNA #3 (sense)	TrueCut Cas9 v2	38	0.98	37
sgRNA #3 (sense)	eSpCas9	49	0.97	48
sgRNA #3 (sense)	SpyFi Cas9	90	0.94	87



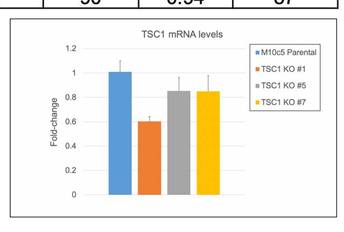
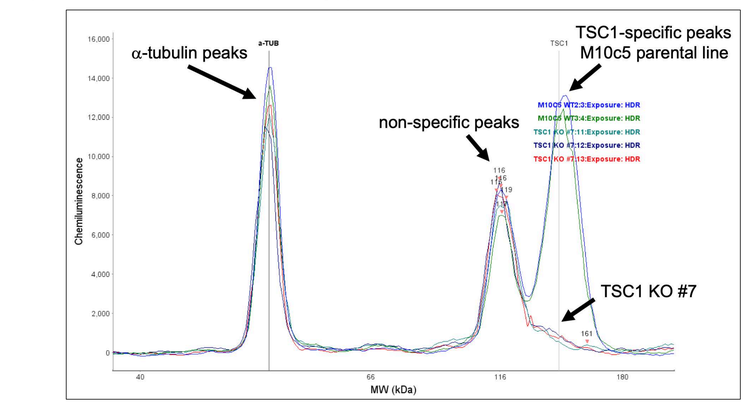


FIGURE 2

TSC1 frameshift mutant clones show complete loss of TSC1 protein expression. TSC1 protein was detected using capillary-based Simple Western blotting. Antibodies against TSC1 and α-Tubulin were multiplexed in the same capillary and detected by chemiluminescence. Computer-generated graphical representation and corresponding quantification are shown below.



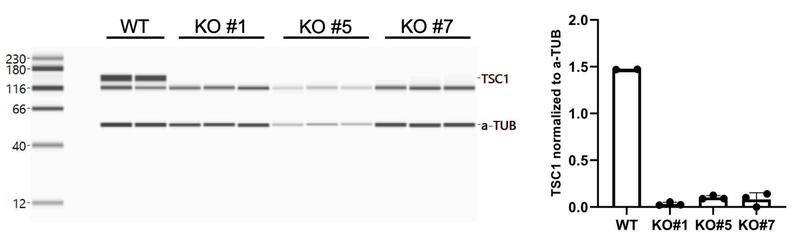


FIGURE 3

TSC1 frameshift mutant clones show upregulation of phospho-p70 S6 kinase. Phosphorylation of p70 S6K at T421/S424 was detected using capillary-based Simple Western blotting. Computer-generated graphical representation and corresponding quantification are shown.

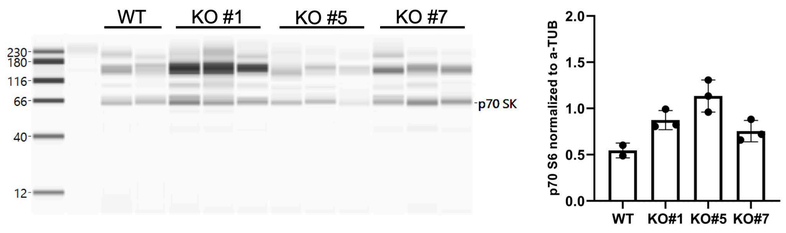


FIGURE 4

Traditional droplet-based single-cell sorting of iPSCs results in poor long-term viability. Live singlet iPSCs can be accurately sorted using BD FACS Aria II system. However, the majority of singlet cells tend to die within the first few days, while others undergo delayed cell death.

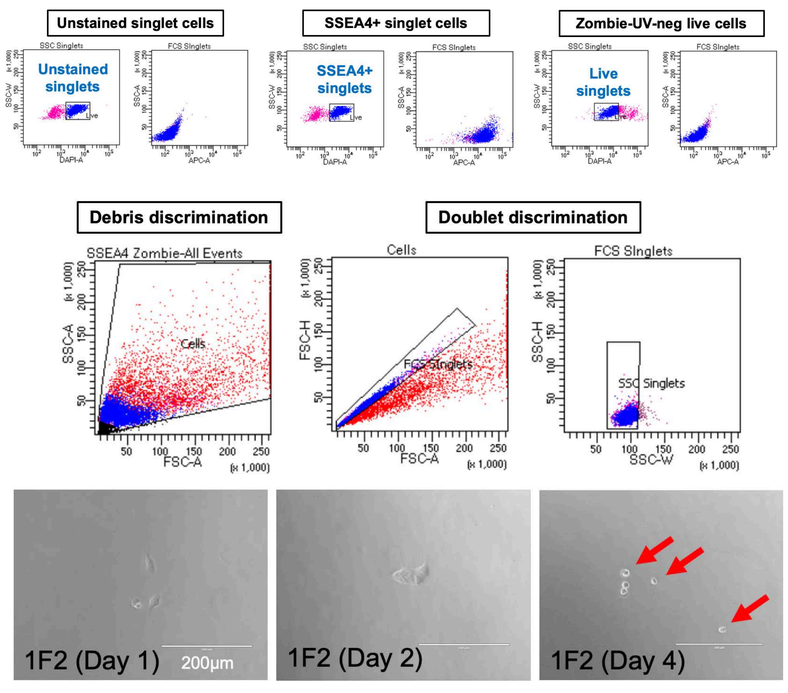


FIGURE 5

A microfluidics-based cell sorter can sort iPSCs as live singlet cells, as well as GFP-positive cells. Untransfected live singlet cells were gated by forward scatter, back-scatter, and 7-AAD exclusion (>665nm emission). Cells were nucleofected with pMAX-GFP plasmid. GFP-positive singlet cells were sorted based on 500-550nm emission.

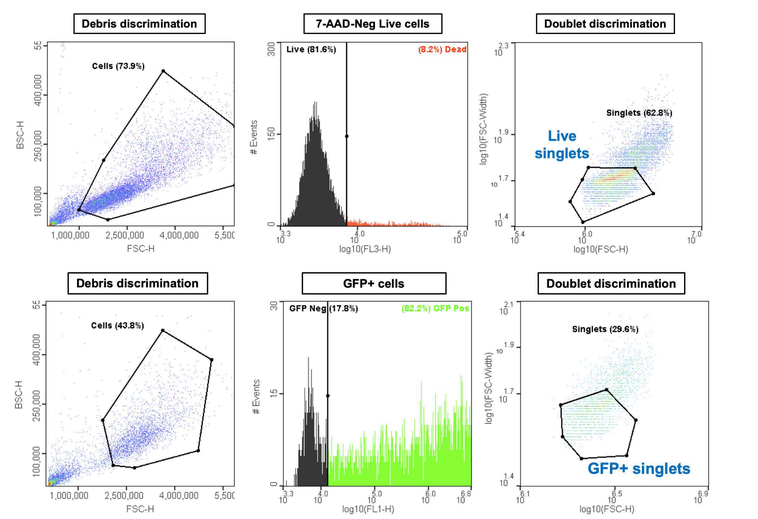
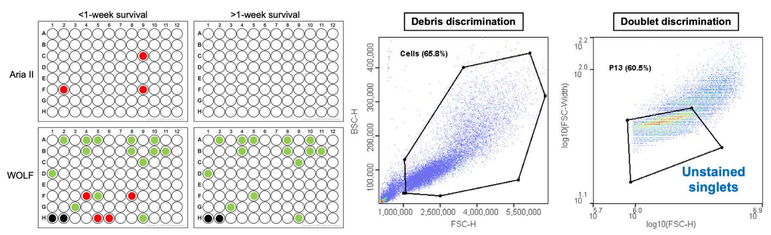
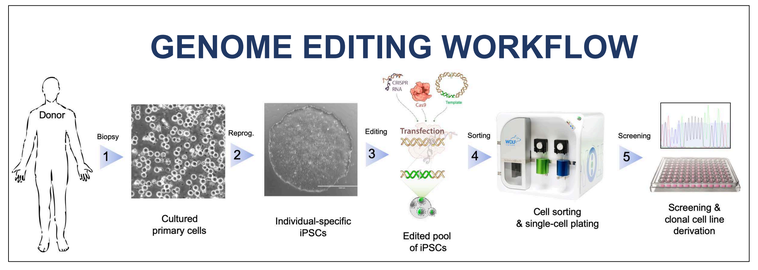


FIGURE 6

Microfluidics-based cell sorting results in better long-term single-cell viability. Edited iPSCs were sorted based on forward scatter and back-scatter properties and monitored for post-sorting survival.





SUMMARY

1. Human iPSCs can be efficiently genome edited by nucleofection of CRISPR/Cas9 RNPs.
2. TSC1 mutant iPS cell lines were created by delivering Cas9 protein and sgRNA targeting a common exon of TSC1. Indels formed by NHEJ were detected in all 8 clones sequenced.
3. Three out of 8 clones contained a biallelic 11-nt deletion, resulting in p.V42GfsTer65.
4. TSC1 frameshift mutants have:
   - reduced mRNA levels
   - undetectable levels of TSC1 protein
   - increased phosphorylation of p70 S6K
5. A microfluidics-based cell sorter can facilitate single-cell cloning of edited iPSCs.

ACKNOWLEDGMENTS

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