

BIG-TREE: Base-Edited Isogenic hPSC Line Generation Using a Transient Reporter for Editing Enrichment

Nicholas Brookhouser,^{1,3,4} Stefan J. Tekel,^{1,4} Kylie Standage-Beier,^{1,2,4} Toan Nguyen,¹ Grace Schwarz,¹ Xiao Wang,^{1,*} and David A. Brafman^{1,*}

¹School of Biological and Health Systems Engineering, Arizona State University, 501 E. Tyler Mall, ECG 334A, Tempe, AZ 85287, USA

²Molecular and Cellular Biology Graduate Program, Arizona State University, Tempe, AZ 85287, USA

³Graduate Program in Clinical Translational Sciences, University of Arizona College of Medicine-Phoenix, Phoenix, AZ 85004, USA

⁴Co-first author

*Correspondence: xiaowang@asu.edu (X.W.), david.brafman@asu.edu (D.A.B.)

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SUMMARY

Current CRISPR-targeted single-nucleotide modifications and subsequent isogenic cell line generation in human pluripotent stem cells (hPSCs) require the introduction of deleterious double-stranded DNA breaks followed by inefficient homology-directed repair (HDR). Here, we utilize Cas9 deaminase base-editing technologies to co-target genomic loci and an episomal reporter to enable single-nucleotide genomic changes in hPSCs without HDR. Together, this method entitled base-edited isogenic hPSC line generation using a transient reporter for editing enrichment (BIG-TREE) allows for single-nucleotide editing efficiencies of >80% across multiple hPSC lines. In addition, we show that BIG-TREE allows for efficient generation of loss-of-function hPSC lines via introduction of premature stop codons. Finally, we use BIG-TREE to achieve efficient multiplex editing of hPSCs at several independent loci. This easily adoptable method will allow for the precise and efficient base editing of hPSCs for use in developmental biology, disease modeling, drug screening, and cell-based therapies.

INTRODUCTION

Current genome editing approaches in human pluripotent stem cells (hPSCs) rely on the introduction of double-stranded breaks (DSBs), which can result in insertion or deletion of DNA sequences, chromosomal translocations, apoptosis, and acquisition of potentially oncogenic mutations (Chapman et al., 2012; Haapaniemi et al., 2018; Ihry et al., 2018; Kosicki et al., 2018). Alternatively, canonical base-editing technologies employ a nicking Cas9 (Cas9^{D10A}) endonuclease fused to a cytidine or adenosine deaminase (Gaudelli et al., 2017; Komor et al., 2016). Base editing does not result in the deleterious byproducts of Cas9 DSB-mediated genome editing and does not require the use of homology-directed repair (HDR) to introduce single base pair changes (Gaudelli et al., 2017; Komor et al., 2016, 2018). In fact, base editors have shown a lower rate of indel formation and fewer off-target editing events than Cas9 (Komor et al., 2018). Over the past several years, various additional base editors have been engineered with different deaminases, targeting windows, editing efficiencies, and PAM specificities (Moon et al., 2019; Rees and Liu, 2018). We recently reported the development of a method called transient reporter for editing enrichment (TREE), which allows for bulk enrichment of base-edited cell populations, including hPSCs (Standage-Beier et al., 2019). Briefly, this method uses a transiently expressed blue fluorescent protein (BFP), which converts to GFP as an assay to report on cytidine deaminase DNA base editor

activity within a cell population. Here, we significantly build upon this work by employing this assay for the highly efficient and rapid production of genetically modified hPSC clonal lines. First, we demonstrate this method, which we entitle base-edited isogenic hPSC line generation using a transient reporter for editing enrichment (BIG-TREE), allows for the rapid generation of clonal isogenic hPSC lines with targeted genome modification frequencies approaching 90%. Next, we establish that BIG-TREE can be utilized in hPSCs for highly efficient introduction of premature stop codons that result in gene loss-of-function. Finally, we demonstrate that BIG-TREE enables efficient multiplex editing of hPSCs in which a high percentage of isolated clones are simultaneously edited at several independent loci. Overall, the easily adoptable methods outlined in this study will significantly advance the implementation of base-editing technologies in hPSCs for researchers interested in developmental biology, disease modeling, drug screening, and regenerative medicine applications.

RESULTS

Highly Efficient Generation of Clonal Isogenic hPSC Lines Using BIG-TREE

In our previous work, we demonstrated that the efficiency at which a base editor is delivered to a cell does not precisely correlate with editing efficiency at a genomic locus.





To overcome this limitation, we developed an assay, termed transient reporter for editing enrichment (TREE) (Standage-Beier et al., 2019). TREE utilizes a BFP variant that converts to a GFP upon a C-to-T nucleotide change (Standage-Beier et al., 2019). More specifically, this BFP mutant contains a histidine at the 66th amino acid position encoded by a “CAC” codon. The C-to-T conversion of that codon to a “TAC” or “TAT” will cause an amino acid change from a histidine to a tyrosine as well as a shift in the emission spectra of the modified protein resulting in a GFP variant. Thus, co-transfection of cells with this BFP construct (pEF-BFP), a base editor (pEF-AncBE4max), and a single guide RNA (sgRNA) targeting the “CAC” codon, sg(BG), will result in a BFP-to-GFP conversion in which the base editor machinery is present and actively functioning. In addition, we found that this BFP-to-GFP conversion was highly predictive of the likelihood of base editing at genomic loci within the same cell that had been transfected with a sgRNA for a genomic target site, sg(TS).

Here, we sought to extend this work to develop a rapid and efficient resource that uses TREE as the basis for the generation of clonal isogenic hPSC lines, termed base-edited isogenic hPSC line generation using a transient reporter for editing enrichment (BIG-TREE) (Figure 1A). As proof-of-principle, we aimed to edit the *APOE* locus, a risk factor associated with altered probability of sporadic Alzheimer disease (AD) onset (Hauser and Ryan, 2013). Human *APOE* has three common isoforms that differ from each other by two amino acids at position 112 and 158 (*APOE2* = Cys112, Cys158; *APOE3* = Cys112, Arg158; *APOE4* = Arg112, Arg158). To this end, we transfected a non-demented control hPSC line (herein referred to as hPSC line 1) that has an *APO* E3/E3 genotype with pEF-BFP, pEF-AncBE4max, and a dual-targeting sgRNA (pDT-sgRNA) vector that contains both sg(BG) and a sgRNA for the *APOE(R158)* locus (Figure 1B, top). Consequently, successful targeting of the *APOE(R158)* locus would result in C-to-T conversion that would cause a change from an *APO* E3 genotype (R158) to an *APO* E2 genotype (C158) (Figure 1C). At 48 h post transfection, fluorescent activated cell sorting (FACS) was used to sort single GFP-positive cells into 96-well plates. Clonal lines were then passaged and expanded over the course of 18 days prior to detailed analysis. First, genomic DNA was isolated from ten clones and the target region of the *APOE* locus, *APOE(R158)*, was subject to Sanger sequencing after PCR amplification (Figure 1C). Remarkably, this analysis revealed that 90% of the clones isolated had been edited, with seven of the clones having a homozygous and two of the clones having a heterozygous edit at the *APOE(R158)* locus (Figure 1D). For comparison, we used a more conventional reporter of transfection (RoT) approach in which this same hPSC line was transfected

with a plasmid in which a GFP and the AncBE4max base editor are driven by the same promoter, connected by a P2A post-translational self-cleavage peptide tag (pEF-AncBE4max-P2A-GFP), as well as the same sgRNA for the *APOE* locus (Figure 1B, bottom). In a manner analogous to that described for the BIG-TREE-based approach, single GFP-positive cells were then sorted into 96-well plates, expanded, and subject to Sanger sequencing. Analysis of ten clonal lines revealed this traditional RoT-based approach was significantly less efficient with only a single clone displaying a heterozygous edit at the target *APOE(R158)* locus (Figure 1D). Given the large variability that exists between individual hPSC lines (Ortmann and Vallier, 2017), we wanted to determine the robustness of BIG-TREE to efficiently generate isogenic pairs in other independent hPSC lines. In this vein, we employed BIG-TREE to target the *APOE* (R158 locus) in two hPSC lines derived from patients with familial AD (FAD) (herein referred to hPSC line 2 and hPSC 3). Analysis of single cell clones by Sanger sequencing (Figure 1C) revealed that across all three hPSC lines tested, over 80% (33/41 clones examined) had an edit at the *APOE(R158)* locus, and greater than 50% of those edits were homozygous in nature (Figure 1E). Importantly, we did not observe the presence of indels at the target site in any of clones examined. Finally, one of the limitations of base editor techniques, regardless if BIG-TREE strategies are employed, is that base editors can induce changes in the protospacer at a C other than the target C within the editing window—termed bystander editing (Figure S1A). Indeed, with respect to generating isogenic lines at the *APOE(R158)* locus, editing at these bystander Cs was a common occurrence (Figure S1B). In fact, only one of the clones analyzed (line 2, clone 5) had a heterozygous edit exclusively at the target C and no other Cs within the editing window. However, it should be noted that these bystander edits did not alter the amino acid sequence.

We performed detailed phenotypic analysis on representative biallelic edited clones from each hPSC line. Overall, these clones had a normal euploid karyotype (Figure 1F), characteristic hPSC morphology (Figure 1G), high expression of key pluripotency markers (Figure 1H), and demonstrated tri-lineage differentiation potential (Figure 1I). In addition, we performed off-target analysis at the top predicted sites for sg(BG) as well as the sgRNA used to target the *APOE(R158)* locus. At all of the off-target sites analyzed, we did not observe any C-to-T conversions at these off-target loci (Figure S2). Furthermore, indels were not identified at any of the off-target sites in clones analyzed. Finally, Sanger sequencing revealed that the AD-related mutations in the hPSC clones derived from the FAD lines were retained in the edited clones (Figure S3). Taken together, this analysis reveals that TREE can be

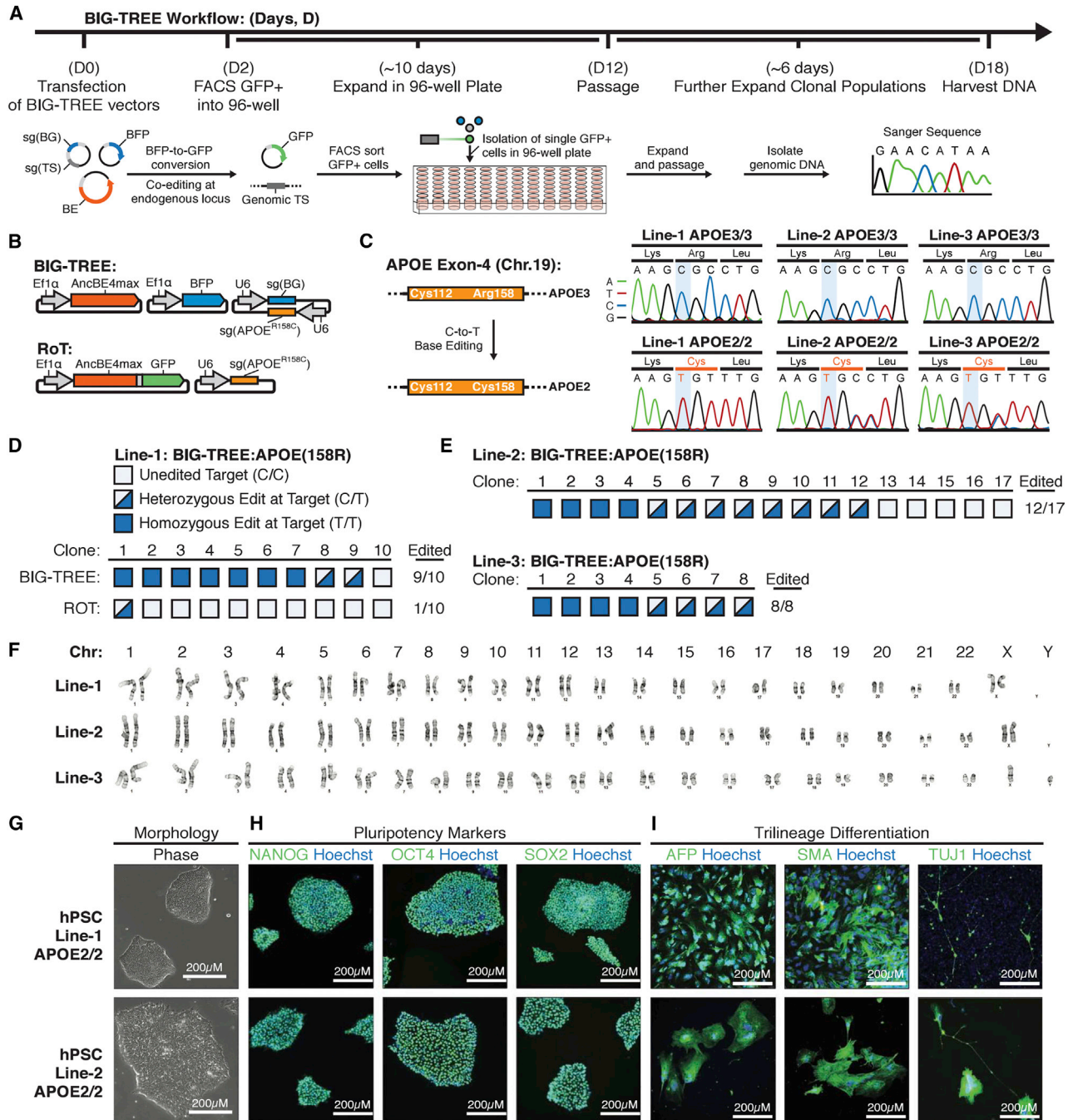


Figure 1. BIG-TREE Enables the Highly Efficient Generation of Isogenic hPSC Lines

(A) Schematic for generation of clonal isogenic hPSC lines using BIG-TREE. HPSCs are co-transfected with pEF-BFP, pEF-AncBE4max, and pDT-sgRNA plasmid vectors. Forty-eight hours post transfection, FACS is used to isolate single GFP-positive cells into 96-well plates. Cells are subsequently expanded, and target clones are identified by Sanger sequencing of the target loci.

(B) Schematic of vectors used for BIG-TREE- and RoT-based generation of clonal hPSC lines in which the APOE(158R) locus has been targeted.

(C) Schematic of the APOE(158R) target locus in exon 4 of the APOE gene. Successful base editing of the APOE(158R) locus would result in a C-to-T conversion causing a change in the amino acid position at 158 from an arginine (APOE3) to a cysteine (APOE2). Representative Sanger sequences of the APOE(158R) locus of unedited parental hPSC lines as well as clonal hPSC lines that have been edited at the

(legend continued on next page)



employed for the highly efficient generation of isogenic hPSCs across multiple independent cell lines.

BIG-TREE Can Be Utilized for the Engineering of Gene Knockout hPSC Lines

To date, engineering of hPSC loss-of-function lines using CRISPR-based approaches has involved the generation of Cas9-mediated DSBs followed by non-homologous end joining (NHEJ), which typically results in a frameshift mutation and introduction of a downstream premature stop codon. Because of the aforementioned caveats associated with such DSB-driven approaches (Chapman et al., 2012; Haapaniemi et al., 2018; Ihry et al., 2018; Kosicki et al., 2018), we wanted to determine if BIG-TREE could be utilized to generate gene knockout hPSC lines without the introduction of DSBs. Because base editors have not been utilized previously to generate loss-of-function in hPSCs, we first wanted to establish this proof-of-principle in HEK293 cells. First, to validate base editor targeted introduction of premature stop codons, we designed a series of sgRNAs targeting an mCherry cassette in an HEK293T line, which would lead to conversion of a “CAG” codon encoding for glutamine to a “TAG” stop codon (Figure S4A). We observed loss of mCherry expression via fluorescent microscopy and flow cytometry when targeting with sgRNAs (Figures S4B and S4C). In addition, we confirmed the targeted addition of stop codons by Sanger sequencing (Figure S4D). Finally, this analysis revealed that loss of mCherry fluorescent signal was a direct consequence of introduction of a premature stop codon introduced into the genomically integrated mCherry cassette (Figure S4E).

Next, we sought to employ BIG-TREE to introduce premature stop codons in hPSCs at a disease relevant locus. To this end, we transfected hPSC line 1 with pEF-BFP, pEF-AncBE4max, and a dual-targeting sgRNA (pDT-sgRNA) vector that contained both sg(BG) and a sgRNA for the glutamine residue at amino acid position 39 in exon 3 of the *APOE* locus. Successful targeting would result in conversion of the glutamine encoding “CAA” codon to a pre-

mature “TAA” stop codon (Figure 2A). Similar to as previously described, we isolated clonal cell lines established from single GFP-positive sorted cells. Analysis of these clones by Sanger sequencing (Figure 2B) revealed that more than 80% of the clones had a stop codon introduced at the target site with greater than 50% of the edited clones displaying a biallelic modification (Figure 2C). Importantly, none of the clones analyzed had indels at the same target site. Lastly, to demonstrate that introduction of a premature stop codon in exon 3 results in functional loss of APOE, we measured the amount of APOE in the conditioned media secreted by unedited and edited cells using ELISA. Compared with the unedited wild-type (Q39/Q39) cells that secreted robust amounts of APOE, cells in which a premature stop codon had been introduced into both alleles (X39/X39) did not secrete any detectable levels of APOE (Figure 2D). Collectively, these data show that BIG-TREE enables efficient generation of loss-of-function hPSC lines through the introduction of premature stop codons.

BIG-TREE Enables High-Frequency, Multiplex Base Editing in hPSCs

Finally, we wanted to determine if BIG-TREE could be utilized with multiplexed genome modification methods to establish hPSC lines that had been simultaneously edited at multiple genomic locations. Accordingly, we utilized a multi-targeting vector (pMT-sgRNA) that contains sg(BG) as well as sgRNAs for three independent genomic target sites (Figure 3A). Analogous to when BIG-TREE was used to target a single genomic location, we employed TREE to simultaneously target multiple loci by co-transfecting hPSC line 1 with pMT-sgRNA, pEF-BFP, and pEF-AncBE4max. Sanger sequencing was then performed on the multiplex targeted genomic sites in clonal hPSC lines derived from single GFP-positive cells (Figure 3B). Along similar lines to when BIG-TREE was used to target a single genomic locus, Sanger sequencing revealed that more than 80% of clones had been targeted at all three sites with all clones displaying biallelic edits (Figure 3C). Moreover, indels

APOE(158R) are shown. Each line shown is representative of clones obtained from three independent parental hPSC populations (hPSC lines 1–3) with different genetic backgrounds.

(D) Distribution of genotypes in clonal hPSCs derived from hPSC line 1 that was targeted at the APOE(158R) locus using BIG-TREE- or RoT-based methods.

(E) Distribution of genotypes in clonal hPSCs derived from hPSC lines 2 and 3 that were generated via BIG-TREE-based targeting at the APOE(158R) locus.

(F) Karyotype analysis of representative clones edited at the APOE(158R) locus.

(G) Phase contrast images of representative clones edited at the APOE(158R) locus.

(H) Immunofluorescence staining of representative clones edited at the APOE(158R) locus for pluripotency markers NANOG, OCT4, and SOX2.

(I) Alpha fetoprotein (AFP), smooth muscle actin (SMA), and beta-III tubulin (TUJ1) immunofluorescence staining of representative clones edited at the APOE(158R) locus that had been subject to tri-lineage differentiation.

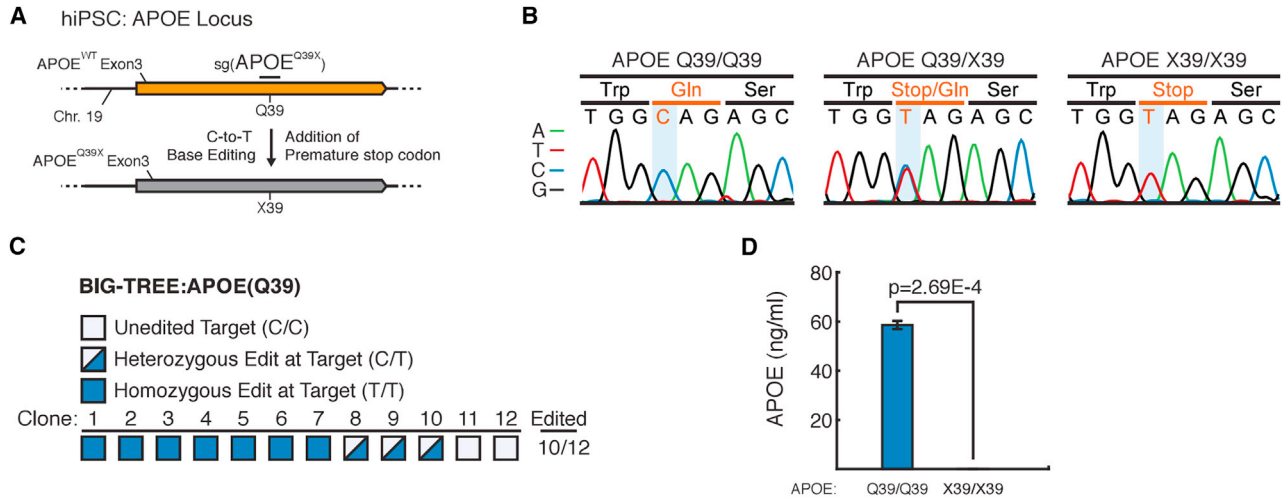


Figure 2. BIG-TREE-Based Gene Knockout of *APOE* in hPSCs

(A) Schematic of the *APOE*(39Q) locus in exon 3 of the *APOE* gene. Successful base editing of the *APOE*(39Q) locus would result in a C-to-T conversion causing a change in the amino acid at position 39 from a glutamine to a premature stop codon. (B) Representative Sanger sequencing of the *APOE*(39Q) locus in unedited wild-type cells (Q39/Q39; left panel) as well as hPSC clones in which a heterozygous (Q39/X39; middle panel) or homozygous (X39/X39; right panel) stop codon has been introduced. (C) Distribution of genotypes in clonal hPSCs that were generated via BIG-TREE targeting the *APOE*(39Q) locus. (D) Measurement of ApoE secretion in the condition medium of wild-type (Q39/Q39) and homozygous edited (X39/X39) hPSCs ($n = 3$ independent experiments, $p < 0.05$, two-tailed Student's *t* test).

were not identified in any of the clones across all three target sites. Lastly, examination of potential bystander edits within the editing window (Figure S1A) revealed a number of clones in which at genomic site 2 and site 3 modification only occurred at the target C and not any other Cs within the editing window (Figure S1C). Specifically, of the ten clones that had homozygous edits at the target C at all three sites, two clones were free from bystander edits at both sites 2 and 3 (clones 1 and 2) and five clones were free from bystander edits at site 3 only (clones 3–7). However, it should be noted that we did not identify any clones in which at genomic site 1 such exclusive modification of the target C occurred. We speculate that because another C occurs immediately adjacent to this target C, that such exclusive modification is likely a rare event that will require site-specific base editors that allow for single-nucleotide changes free from bystander editing at adjacent nucleotides (Tan et al., 2019).

DISCUSSION

In summary, we establish that BIG-TREE is a fast and efficient protocol for the generation of clonal isogenic hPSC lines with homozygous and heterozygous single base pair edits. Because the number of diseases that are a consequence of single point mutations (Landrum et al., 2018), as well as the growing number of genomic variants

of uncertain significance that have been identified through large-scale sequencing efforts (Kobayashi et al., 2017), the ability to rapidly engineer isogenic hPSC lines will have a significant impact on the establishment of *in vitro* models to assess pathogenic risk and dissect disease-causing mechanisms. In addition, in this study, we demonstrate that BIG-TREE can be employed to generate effective loss-of-function cell lines through the introduction of premature stop codons. Currently, most CRISPR/Cas9-based approaches to generate gene knockouts involve the introduction of deleterious DSBs followed by NHEJ-mediated repair that results in frameshift and loss of gene function (Carlson-Stevermer and Saha, 2017; Ihry et al., 2019). As we describe in this study, the ability to rapidly generate gene knockouts without the need for DSBs will have important implications for the use of hPSCs to elucidate the function of specific genes in development and disease. Lastly, we establish that BIG-TREE can allow for the generation of clonal hPSC lines that have been simultaneously edited at multiple independent loci, an important consideration given that many diseases are polygenetic in nature (Khera et al., 2018). By comparison, conventional CRISPR/Cas9-based approaches are too inefficient in hPSCs to employ multiplexing editing strategies.

Since the first base editors were engineered (Komor et al., 2016), numerous additional base editors with targeting windows, editing efficiencies, PAM specificities, and

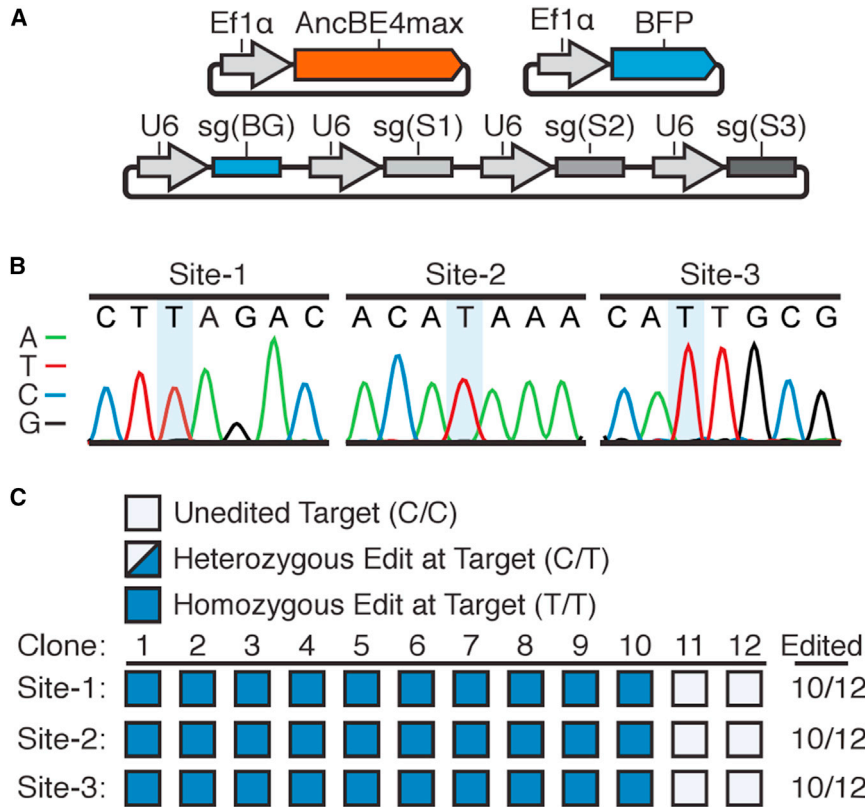


Figure 3. BIG-TREE Allows for Simultaneous Base Editing of Multiple Loci in hPSCs

(A) Schematic of plasmid vectors used for BIG-TREE-based generation of clonal hPSC lines in which multiple loci have been simultaneously targeted. The pMT-sgRNA vector contains sg(BG) in addition to sgRNA for multiple target sites (S1, genomic site 1; S2, genomic site 2; S3, genomic site 3).

(B) Representative Sanger sequencing chromatographs of the site 1, site 2, and site 3 loci in clonal hPSCs that have been generated via BIG-TREE multiplexed base editing.

(C) Distribution of genotypes in clonal hPSCs that were generated via BIG-TREE multiplexed base editing.

deaminases have been generated (Moon et al., 2019). In the context of BIG-TREE, we employed AncBE4max, which displays a relatively high editing efficiency with low off-target activity (Koblan et al., 2018). However, one of the limitations of AncBE4max is that it can induce C-to-T conversions at bystander Cs within the editing window. Although bystander editing was a common occurrence in our clonal populations, we did observe clones with exclusive modifications of the target C. More specifically, when generating isogenic lines edited at the APOE(R158) locus, we only isolated one clone that had a monoallelic edit exclusively at the target C. Nonetheless, all of the bystander edits that we observed at the APOE(R158) locus did not impact the amino acid sequence, mitigating the impact on the downstream application of these hPSC lines. With regard to the multiplex editing, we did observe several clones that were free from bystander edits at genomic sites 2 and 3. However, at genomic site 1, where a C is present in the base pair position directly next to the target C, we did not isolate any clones where modification only occurred at the target C. In the future, given the ease of use, we anticipate that utilizing BIG-TREE with these other base editor variants with a narrow editing window will be easily achieved. In this regard, the end-user can select to employ such base editors with a more stringent editing window if

editing at a bystander C is not tolerable (e.g., results in changes in the amino acid coding sequence).

In general, there are several enabling aspects to the methods presented in the study that will allow for the facile adoption by a broad set of researchers. First, the high editing frequencies do not require the screening of large numbers of clones to identify those with the desired modification. Moreover, we demonstrate that BIG-TREE is robust, as it allows for the efficient editing of multiple loci and across several independent hPSC lines. Because of these efficiencies, clonal lines can be identified, expanded, and characterized in the course of a few weeks. Along similar lines, the high efficiency of BIG-TREE allows for the biallelic or multiplexed targeting without the need for sequential re-targeting. In addition, BIG-TREE is compatible with off-the-shelf chemical transfection reagents and does not require the cloning of complex viral constructs or the use of specialized cell transfection systems. In fact, all sgRNA vectors were designed to allow for the facile cloning of new target sites via BbsI restriction enzyme digestion and ligation of oligonucleotides that target the desired genomic sequence. Lastly, BIG-TREE offers the flexibility to be used in conjunction with other base editor variants that have altered PAM specificities and editing windows (Huang et al., 2019; Tan et al., 2019;



Thuronyi et al., 2019). In conclusion, we contend that BIG-TREE is a readily adoptable method that will enhance and accelerate the use of base-editing approaches in hPSCs.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions

Cell lines, media compositions, and conditions for culture of hPSC and HEK293 are listed in the [Supplemental Experimental Procedures](#).

Plasmid Construction

All plasmids were constructed using conventional restriction enzyme-based molecular cloning techniques. For construction of the sgRNA plasmids, the sgRNA sequences listed in [Table S1](#) were used. Additional details for molecular cloning and plasmid construction are provided in the [Supplemental Experimental Procedures](#).

hPSC Base Editing, Clonal Isolation, and Characterization

Methods for transfection of hPSCs, clonal isolation, and characterization via tri-lineage differentiation are described in the [Supplemental Experimental Procedures](#).

Genotyping and Sequence Analysis at Off- and On-Target Analysis

Genomic DNA was prepared from expanded clones using the DNeasy kit (QIAGEN). PCR was performed with the primers listed in [Table S2](#) using the methods described in the [Supplemental Experimental Procedures](#).

Karyotype Analysis

For each cell line, cytogenetic analysis was performed (Cell Line Genetics) on 20 metaphase cells using standard protocols for G-banding.

Immunofluorescence

Detailed protocols for immunofluorescence are provided in the [Supplemental Experimental Procedures](#). Antibodies used are listed in [Supplemental Experimental Procedures](#).

HEK293 Transfections

Methods for transfection of HEK293s and Sanger sequencing of resultant populations are described in the [Supplemental Experimental Procedures](#).

Fluorescence Microscopy

Fluorescent imaging was performed on a Nikon Ti-Eclipse inverted microscope using the filters and acquisition settings described in the [Supplemental Experimental Procedures](#).

Flow Cytometry

Cells were dissociated with Accutase for 10 min at 37°C, triturated, and passed through a 40 µm cell strainer. Cells were then washed

twice with flow cytometry buffer (BD Biosciences) and resuspended at a maximum concentration of 5×10^6 cells per 100 µL. Flow cytometry analysis was performed on an Attune NxT (Thermo Fisher Scientific). Flow cytometry files were analyzed using FlowJo (FlowJo LLC, Ashland, OR, USA).

Apolipoprotein E ELISA

Cells were seeded in a 6-well plate at a density of 3×10^5 cells per well. Medium was changed every 24 h. On day 3, 24-h conditioned medium was collected, and ApoE levels in the medium were measured with the Human APOE (AD2) ELISA Kit (Thermo Scientific).

Statistical Analysis

Unless otherwise noted, all data are displayed as means \pm SD.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.stemcr.2019.12.013>.

AUTHOR CONTRIBUTIONS

N.B., S.J.T., K.S.B., X.W., and D.A.B. designed the experiments and wrote the manuscript. X.W. and D.A.B. supervised the research. N.B., S.J.T., K.S.B., T.N., and G.S. performed the experiments and analyzed the data.

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