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A stress-free strategy to correct point mutations in patient iPS cells

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ABSTRACT

When studying patient specific induced pluripotent stem cells (iPS cells) as a disease model, the ideal control is an isogenic line that has corrected the point mutation, instead of iPS cells from siblings or other healthy subjects. However, repairing a point mutation in iPS cells even with the newly developed CRISPR-Cas9 technique remains difficult and time-consuming. Here we report a strategy that makes the Cas9 “knock-in” methodology both hassle-free and error-free. Instead of selecting a Cas9 recognition site close to the point mutation, we chose a site located in the nearest intron. We constructed a donor template with the fragment containing the corrected point mutation as one of the homologous recombination arms flanking a PGK-Puro^R cassette. After selection with puromycin, positive clones were identified and further transfected with a CRE vector to remove the PGK-Puro^R cassette. Using this methodology, we successfully repaired the point mutation G2019S of the *LRRK2* gene in a Parkinson Disease (PD) patient iPS line and the point mutation R329H of the *AARS1* gene in a Charcot-Marie-Tooth disease (CMT) patient iPS line. These isogenic iPS lines are ideal as a control in future studies.

1. Introduction

About half of the known human pathogenic genetic variants are point mutations (Anzalone et al., 2019) in which the disease gene only has one nucleotide difference in patients compared with that of healthy individuals. Thus, a strategy which uses an easy and reliable way to correct point mutations would represent a significant step forward. Recent developments in gene targeting techniques, especially CRISPR-Cas9, makes routine the repair of these genetic mutations (Protocols like in (Ran et al., 2013)). In such applications, synthesized single-stranded oligodeoxynucleotides (ssODN) as donor templates are most commonly used in homology-directed repair-based (HDR) knock-in for small repairs (Richardson et al., 2016). The knock-in efficiency has been shown to be higher when using ssODNs with the introduction of a silent mutation at the PAM site to prevent cutting on the edited allele (Armstrong et al., 2016). Also using asymmetric donor DNA can increase the HDR efficiency (Richardson et al., 2016). However, several obstacles still remain. First, finding suitable Cas9 cleavage sites is not always practical. The conventional CRISPR-Cas9 “knock-in” strategy usually requires that the selected cleavage site resides as close as possible to the mutation site to achieve higher efficiency (Bialk et al., 2015; Harmsen

et al., 2018; Paquet et al., 2016). Secondly, while double-strand breakage has a higher efficiency of HDR than single-strand nicks, many non-homologous end joining (NHEJ) events happen during double-strand breakage. Moreover, using synthesized ssODN with short homologous arms produces more occurrences of insertions and deletions (indels) or point mutations compared to plasmid donor templates with long homologous arms (Elliott et al., 1998). Most critically, the efficiency of homologous repair is still low even with the latest development of RNP (ribonucleoprotein: synthetic sgRNA and Cas9 recombinant protein complex) (Okamoto et al., 2019) when there is no reporter or selection cassettes. Finally, in order to identify positively targeted clones, it is necessary to sequence the genomic components of a large number of clones.

To repair point mutations more efficiently, the Liu lab first developed the base editor using a catalytically impaired Cas protein (dCas or Cas nickase) connected with a DNA-modifying enzyme, a deaminase to make precise base substitution possible. After sgRNA directs dCas to the target site, without double-strand cleavage or donor template DNA, the deaminase modifies the base instead of Cas9 cleavage of the DNA (Komor et al., 2016). Current base editing techniques only allow base substitutions of C•G to T•A that is mediated by cytosine base editors

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(CBEs), and base substitutions of A•T to G•C that is mediated by adenine base editors (ABEs). Consequently, the speed of base editing can be slow and the selection of the targeted site limited.

Here, we apply the CRISPR-Cas9 technique to generate an isogenic line from the most common variant of *LRRK2* gene in both familial and idiopathic PD patients (Goldwurm et al., 2005). This c.6055G > A point mutation changes Gly to Ser at 2019aa, increasing kinase activity of LRRK2 (West et al., 2005). It remains unclear why this activated form of LRRK2 is tightly associated with PD. In the last decades, iPS cell tech-

extracted from iPS cells of normal subject. Subsequently, they were cloned into the human Oct4-GFP vector (Addgene Plasmid #21153) replacing the Oct4-L arm-2A-GFP and Oct4-R arm and were sequenced to confirm correct amplification and connection. All cloning primers are listed below. The underlined nucleotides of LRRK2-R arm primers are the restriction enzyme recognition sites. The other three arms were cloned using NEB Hi-Fi DNA Assembly kit (New England Biolabs) due to the lack of suitable restriction enzyme sites. The underlined nucleotides therein marked the sequences of the vector.

Fragment Name	Forward Primer Sequence	Reverse Primer Sequence
LRRK2-L arm	<u>caagcttggtaccgagctcg</u> CCTTAATATCTAACATGATTAGGTTTATG	<u>gggttattgaatatgatcg</u> gAAGATAGAATTATGAGACAGAC
LRRK2-R arm	TTTATTGGCGCGCCAGGATGGATAACCACTGAC	TTTATTGGCGCGCTCCCTAAAGATAGAGTGTTC
AARS1-L arm	<u>caagcttggtaccgagctcg</u> AATGGCCGATCTTGCTC	<u>gggttattgaatatgatcg</u> gAAGCAACACCTCTTTCAGGAAC
AARS1-R arm	<u>cctgaagaggttcactagg</u> GGGTAGGATCCAAAGGGAC	<u>ctctagatgcatgctcgagc</u> TCTGTGAGAAAGGGCTTG

nology brought about a revolutionary change in human disease modeling. Thus, with the introduction of a combination of 3 or 4 transcription factors, somatic cells can be transformed into stem cells with unlimited capacity for cell division and differentiation (Takahashi et al., 2007). Reprogrammed patient cells can be used to explore disease mechanisms or screen for possible useful drug therapies. In these studies, the use of control isogenic cell lines derived from the same patient iPS cells is critical. Instead of designing the sgRNA recognition site close to the mutation site, we chose a location in the nearest intron that gives more flexibility to choose an optimal sgRNA location. Having the corrected sequence on one of the homologous arms, the mutated allele was repaired through homologous recombination. We also included a loxP flanked PGK-PuroR cassette so that after selection with puromycin, the efficiency of choosing positive clones would be much higher. With this new efficient strategy, we successfully obtained clones that have corrected the point mutation. Similarly, using this same gene editing strategy, we also efficiently repaired the c.986G > A point mutation of *AARS1* gene, which changes Arg to His at 329aa in familial CMT patients (McLaughlin et al., 2012). The *AARS1* gene codes for the growth-dependent essential enzyme alanyl-tRNA synthetase, which catalyzes aminoacylation of tRNA^{Ala} with alanine to support translation of alanine codons during protein synthesis on the ribosome. The R329H mutation of *AARS1* severely compromises the catalytic activity of the enzyme (McLaughlin et al., 2012), emphasizing the importance to study this mutation in the development of the CMT disease.

2. Materials and methods

2.1. Cell culture

LRRK2 (ND40018**C*) iPS cell line was obtained from NINDS human genetics DNA and cell line repository. Fibroblasts harboring the R329H *AARS1* mutation were isolated from an anonymous patient in Australia through clinical collaboration with Dr. Michael Shy of University of Iowa, and were made into 100550A iPS cell line with mRNA reprogramming kit (Stemgent). BJ fibroblast were obtained from ATCC (CRL-2522™) and reprogrammed with the same mRNA reprogramming kit. LRRK2 and BJ iPS cells were maintained in mTeSR1 medium (Stem Cell Technologies) and 100550A iPS cells were kept in Stemflex medium (Thermo-Fisher) at 37 °C in a 5% CO₂ incubator. Cells were passaged using 0.5 mM EDTA and replated onto Cultrex (1:100 diluted in DMEM/F12, Biotechne)-coated plates.

2.2. Construction of donor plasmids

For generating LRRK2-PGK-Puro and AARS1-PGK-Puro vectors, two pairs of homologous arms, LRRK2-L and LRRK2-R arms, and AARS1-L and AARS1-R arms, were amplified from human genomic DNA

2.3. Gene targeting in hiPSCs

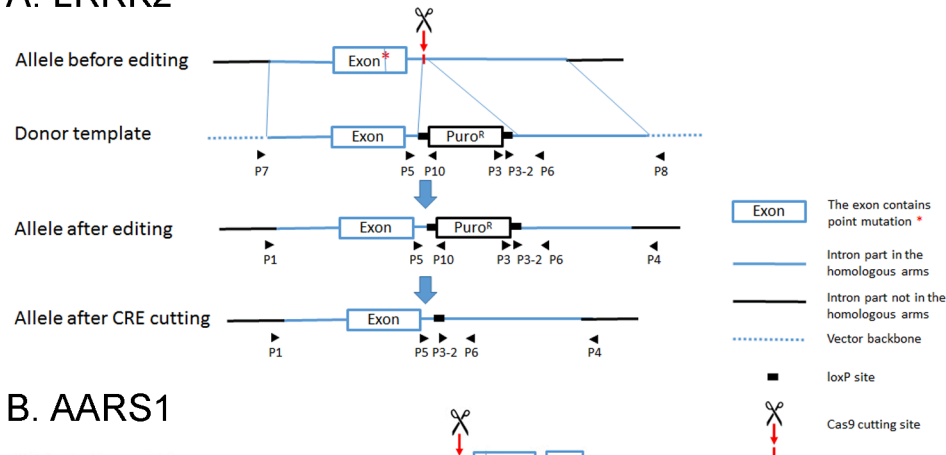
A million of LRRK2 or 100550A iPS cells were harvested using Accutase (Sigma) and reverse-transfected with 1 µg of donor construct, 12 pmol spCas9 protein (Aldevron), and 18 pmol of sgRNA (*LRRK2*: 5'-GAAGTACATCTGAGGTCAG-3', *AARS1*: 5'-GGGCGTATCGGACAGCTCGG-3', Synthego), 4 µl P3000 reagent and later with 5 µl Lipofectamine 3000 (Thermo-Fisher). A mixture of transfection reagents was added onto a Cultrex-coated well first and then followed by resuspended LRRK2 iPS cells or 100550A iPS cells in fresh medium with 5 µM Y-27632 (Stemgent). Puromycin (500 ng/ml, Sigma) was added into the medium three to five days after transfection. Drug-resistant cells were replated at low density (5,000 cells/100 mm dish) and single cell colonies were manually selected afterwards. Clones with both 5' and 3' insertion positive genotyping results were further expanded and the puromycin cassette was deleted by transient transfection of a CRE vector pCAG-Cre:GFP (Addgene #13776) and then plated at low density for single cell colonies. After a 2nd round of genotyping, positive clones were expanded and characterized.

2.4. Genotyping

Cells were collected and treated with 1x lysis buffer in PBS (For 4x stock: Tris-HCl pH 8 (10 mM), Triton X (2%), EDTA (4 mM) and freshly added Proteinase K (1%)) at 60 °C for 1 hr followed by 95 °C for 10 min. Genotyping was done using 1 µl of the lysis mixture as templates and two sets of primers to confirm both the 5' insertion and the 3' insertion at the first round. Primer set LRRK2-P1, AARS1-P1 and Puro-pA-R-P10 were used for confirmation of the 5' insertion. Primer set Puro-pA-P3-2 and LRRK2-P4, AARS1-P4 were used for confirmation of the 3' insertion. A primer set P1 and P6 was used to distinguish between WT (946 bp) and targeted alleles (1042 bp) at the second round of *LRRK2* targeting. The genomic DNA amplified by the primer set LRRK2-P5 and LRRK2-P6 was sent for sequencing. Primer set of AARS1-P5 and AARS1-P6 was used to distinguish between WT (189 bp) and targeted alleles (275 bp) at the second round of *AARS1* targeting.

Primer Name	Primer Sequence
LRRK2-P1	ATTGCGTGGGTCAGTCTC
Puro-pA-P3	GCAGCCTCTGTTCACATAC
Puro-pA-P3-2	CCCTCGAAGAGGTTCACTAG
LRRK2-P4	AGCAAGATGATGTATAGCCACC
LRRK2-P5	TAAGGGACAAAGTGAGCACAG
LRRK2-P6	ATCTGAGGTCAGTGGTTATCC
Puro-pA-R-P10	GACGTAACCTCTCTTCAGACC
AARS1-P1	ACGTAGCTGGGATTATAGGTG
AARS1-P4	CAGAGACATGAGAGCCCAAC
AARS1-P5	TGACCTTGGGGAATGCGAAA
AARS1-P6	TTCATGGGCGTATCGGACAG

A. LRRK2



B. AARS1

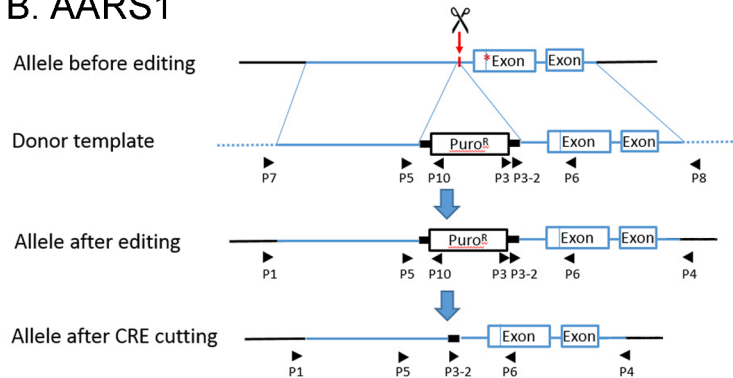


Fig. 1. A stress-free strategy of correcting point mutations in patient iPSCs. A, Schematic representation of the strategy to generate a *LRRK2* isogenic line with the corrected allele (correction of the c.6055G > A mutation of *LRRK2* gene). B, Schematic representation of the strategy to generate a 100550A isogenic line with the corrected allele (correction of the c.986G > A mutation of *AARS1* gene).

2.5. QX200 Droplet Digital PCR (ddPCR™, BIO-RAD)

Genomic DNA was purified from each targeted 100550A cell sample with QIAamp DNA Mini Kit (Qiagen). A 20 µl PCR reaction mixture containing 50–100 ng genomic DNA, primer and probe set (designed by BIO-RAD, Cat# 10049047, Project dHsaMDS305174770), and QX200™ ddPCR™ Supermix for probes (no dUTP) (BIO-RAD) were used for droplet generation. Emulsified samples were then transferred to PCR plates according to manufacturer's instructions. The cycling protocol was used with a 95 °C enzyme activation step for 5 min followed by 40 cycles of a two-step cycling protocol (95 °C for 30 s and 55 °C for 1 min). The ramp rate between these steps was 2 °C/second. The post cycling step of enzyme deactivation was set at 98 °C for 10 min with the ramp rate 1 °C/second. After PCR reaction, the plate was put in the QX200™ Droplet Reader and the events were recorded using the program QuantaSoft.

2.6. Off-targeting analysis

LRRK2 and *AARS1* sgRNA sequences were submitted to the website <http://www.rgenome.net/cas-offinder/> to search for possible off-targeting human genomic positions with the threshold of 4 mismatches. All resultant genomic fragments (217 for *LRRK2* and 11 for *AARS1*) were then blasted to the database: Human genomic plus transcript (Human G + T) at <https://blast.ncbi.nlm.nih.gov/Blast.cgi>. There was only one DNA fragment (similar to *LRRK2*) matching the coding area of human transcripts *ANXA9* and one DNA fragment (similar to *AARS1*) matching the coding area of human transcripts *PTX4*. The corresponding genomic region of *ANXA9* was amplified by the primers from both isogenic and parental *LRRK2* lines (*ANXA9*-F: CCGTGATGGTACTTGTGCCT; *ANXA9*-R: CATCTTATGTGCATGGCGGC). The corresponding genomic region of *PTX4* was amplified by the primers from

both isogenic and parental 100550A lines (*PTX4*-F2: ATCCAATTTCGTGATCGGGGA; *PTX4*-R2: GCCCTTGCTGGCCTCAG). Then the amplicons were sequenced to investigate any changes for those areas.

2.7. Immunocytochemistry

Undifferentiated isogenic iPSCs were fixed with 4% paraformaldehyde in 1x PBS for 20 min and blocked with 1% (v/v) bovine serum albumin (Sigma), 0.2% (v/v) Triton-X (SigmaAldrich), 5% normal donkey or goat serum in 1 × PBS for 30 min. They were then incubated with primary antibodies overnight at 4 °C, and subsequently labeled with fluorescence-conjugated secondary antibodies for 1 h. Images were acquired using a fluorescence microscope (Olympus Optical, JP/IX-71).

2.8. STR analysis

Short tandem repeat (STR) analysis was performed on generated isogenic *LRRK2* and 100550A clones, together with their parental clones using the PowerPlex®16 System (Promega) with the detection of 16 allele loci at the Molecular & Genomic Pathology Laboratory of Thomas Jefferson University Hospital.

3. Results and discussion

3.1. Cas9 and sgRNA can target the *LRRK2* locus in the *LRRK2* iPS line and *AARS1* in the 100550A iPS line

In order to correct the point mutation c.6055G > A of the *LRRK2* gene and c.986G > A of the *AARS1* gene, we chose the Cas9 recognition site in the intron closest to each mutation. For *LRRK2* gene, it was in the intron after the exon containing the mutation. For *AARS1* gene, it was in

A LRRK2 isogenic clones after CRE

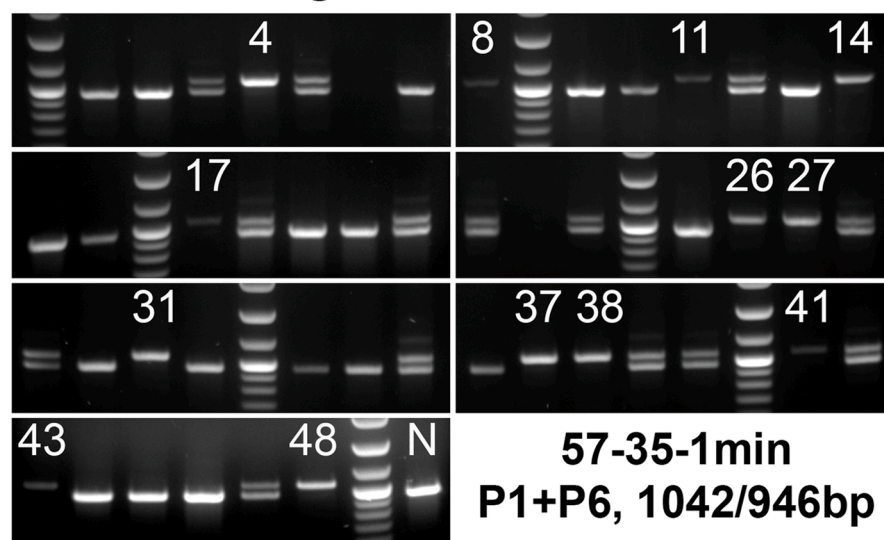
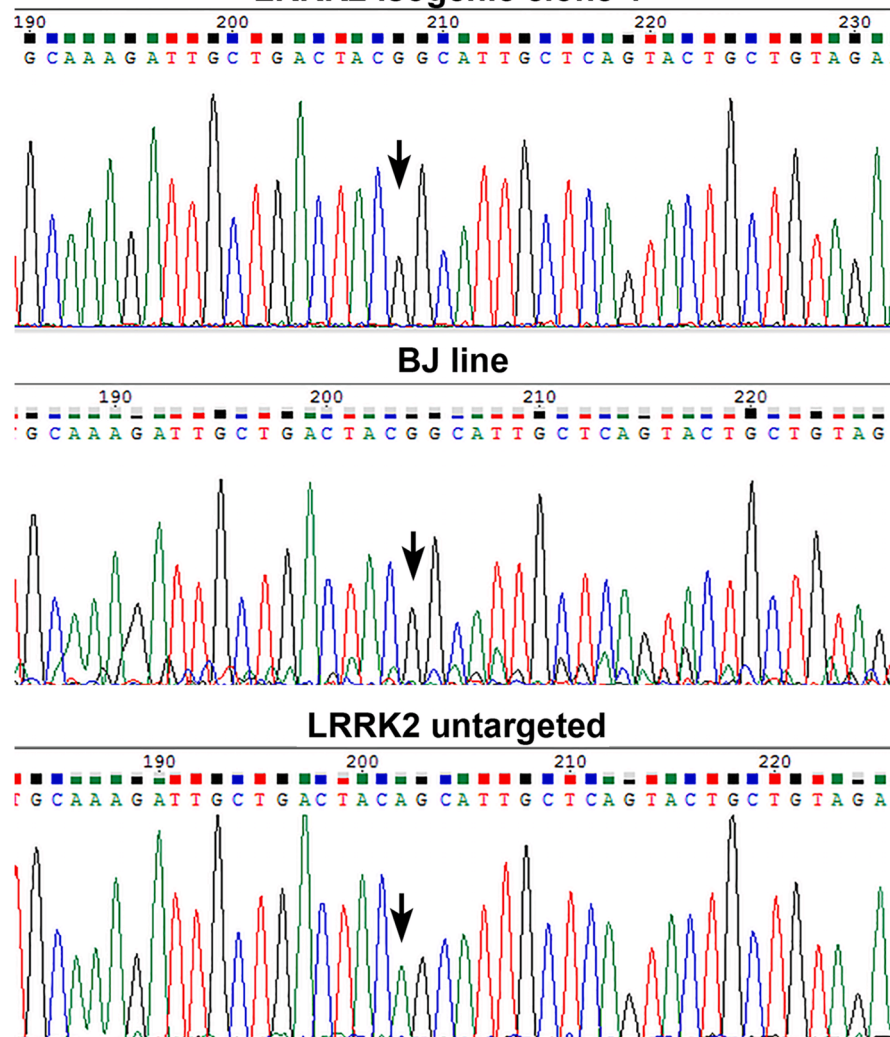


Fig. 2. Correction of the c.6055G > A mutation in *LRRK2*. A, Genotyping results of 48 *LRRK2* isogenic clones after CRE cleavage of the PGK-Puro^R cassette. The PCR primer set P1 + P6 was used to amplify the region covering the exon and the cutting area of the intron. When one allele was edited, the remaining loxP site made the amplicon (1042 bp) larger than that of the unedited allele (946 bp). The genotyping PCR was performed with 57 °C annealing temperature, 35 cycles and 1 min for extending step. The clone numbers are labeled for those clones having both alleles targeted, which expressed as one single up-shifted band. N marks untargeted *LRRK2* cells expressing only the untargeted band as a negative control. B, Sequencing results of the G > A mutation area of a homozygous *LRRK2* isogenic clone 4 line (top row). BJ line, an iPS cell line from a healthy subject with the WT *LRRK2* gene was used as a positive control (middle row). An unedited *LRRK2* parent line was used as a negative control (bottom row). The arrows are pointing to the nucleotide of interest.

B LRRK2 isogenic clone 4



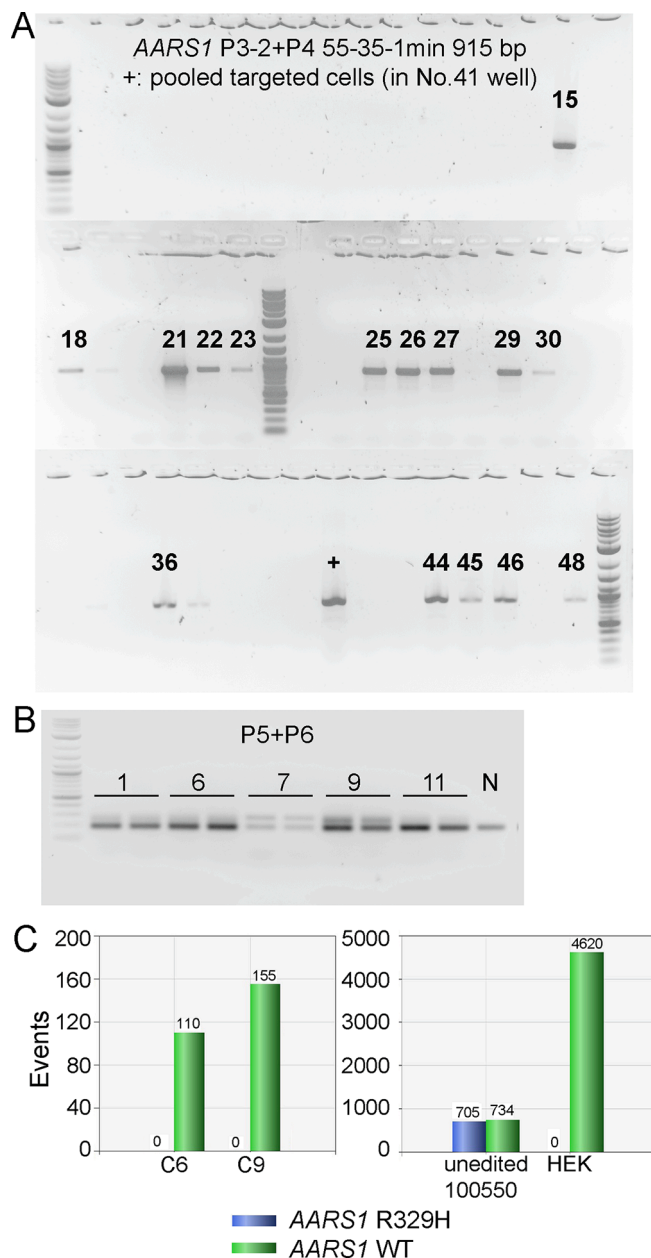


Fig. 3. Correction of the c.986G > A mutation in *AARS1*. **A**, Genotyping results of 47 100550A clones after puromycin selection. Using the PGK-Puro^R cassette specific primer P3-2, the clones with the amplified band (915 bp, as shown) were the targeted clones. Pooled cells after selection were used as a positive control (+). This genotyping PCR was performed with 55 °C annealing temperature, 35 cycles and 1 min for extending step. **B**, Genotyping results of 5 positive clones (Clone No: 1, 6, 7, 9, 11) after CRE removal. P5 + P6 amplification produced two bands in each of the 5 clones, all of which were heterozygotes with one targeted allele and one untargeted allele. Negative (N) control was unedited 100550A iPS cells. This genotyping PCR was performed with 55 °C annealing temperature, 35 cycles and 30 s for extending step. **C**, Genotyping of 2 clones using ddPCR. There were only amplification from wild type primer set and probe in Clone 6 (C6) and Clone 9 (C9). Unedited 100550A iPS cells were used as a positive control, showing a similar positive number of events of both wild type and mutant *AARS1* gene. HEK293 cells were used as a negative control for mutant *AARS1* gene.

the intron in front of the exon. To design the sgRNA, the first 100 bp of the intron in human *LRRK2* gene or the last 100 bp of the intron of *AARS1* gene was analyzed to identify suitable target sites using the designing tool from Broad Institute (<https://portals.broadinstitute.org/gpp/public/analysis-tools/sgRNA-design>).

Fig. 1 describes our targeting strategy for correcting the point mutation using Cas9-mediated homologous recombination. The donor template contains the corrected *LRRK2* gene fragments (A) or the corrected *AARS1* gene fragments (B) amplified from a normal subject iPS line, BJ, spanning the pGK-Puro^R cassette. Following sequencing analysis and confirmation of the successful cloning of donor template, the RNP and donor template was reverse transfected into the *LRRK2* or 100550A iPS cells with Lipofectamine 3000. After puromycin selection, surviving cells were genotyped. Nine of 45 *LRRK2* clones showed positive for both the 5' insertion (P1 + P10) and the 3' insertion (P3-2 + P4) (Data not shown). These successful gene targeted clones were pooled together and were further transfected with a CRE vector pCAG-Cre:GFP to remove the selection cassette. Then single cell colonies were manually selected and genotyped for the second time. From the screening results of PCR with primers P1 and P6, 13 of the 48 clones exhibited only an up-shifted band, indicating positive gene targeting of both alleles (**Fig. 2A**). After sequencing each of these up-shifted bands, we successfully confirmed that the correction of both alleles was made in these *LRRK2* isogenic clones (**Fig. 2B**). Fifteen of 47 100550A clones demonstrated positive for the 3' insertion (P3-2 + P4) (**Fig. 3A**). In contrast to the *LRRK2* isogenic cells, the 100550A isogenic clones only contained one allele targeted while the other allele unaffected. Consistently, PCR screening with primer set P5 + P6 revealed no single band in each clone tested (**Fig. 3B**). To further confirm that the mutation was repaired, genomic DNA from the positive clones was purified and analyzed using ddPCR primers and two probes detecting either wild type or mutant alleles. Indeed, two clones tested showed no mutant allele (**Fig. 3C**), indicating that the mutation had been corrected to the WT sequence.

The confirmed *LRRK2* isogenic clones (**Fig. 4 A-D**) and 100550A isogenic clones (data not shown) were evaluated for pluripotency markers including Oct3/4, Sox2, Nanog, and SSEA-4 to confirm their cell stemness. The results showed that these isogenic clones maintained expression of their pluripotency markers. In addition, the genomic DNA of *LRRK2* isogenic clone 4 and 100550A isogenic clone 9, together with that of the respective parent lines, was sent for Short Tandem Repeat (STR) analysis. The genomic DNA of both isogenic clones exhibited the same patterns for all sixteen markers examined as those of their respective parent lines (**Fig. 4E**), demonstrating that both lines of isogenic clones originated from the respective parent iPS lines. We checked both *LRRK2* and *AARS1* sgRNA for potential off-targeting events on the website <http://www.rgenome.net/cas-offinder/>. The results returned with 16 genomic regions of 3-mismatches and 201 of 4-mismatches for *LRRK2* sgRNA, one 3-mismatch and ten 4-mismatches for *AARS1* sgRNA. After blasting all resultant DNA sequences at NCBI, we found only one match (*LRRK2* sgRNA sequence) to the coding area of *ANXA9* gene and one match (*AARS1* sgRNA sequence) to the coding area of *PTX4* gene. The majority of other genomic DNA regions were not matching to any human transcripts with the exception of a couple of DNA sequences found on some transcripts but outside the coding area (Excel file submitted as **Supplementary data**). With specific primers, we amplified the matching *ANXA9* region from both *LRRK2* isogenic and parental iPS lines and the matching *PTX4* region from both 100550A isogenic and parental iPS lines. After sequencing all 4 DNA fragments, we found no changes in the area of *ANXA9* gene or *PTX4* gene (**Figure Supplementary figure 1**, **Figure Supplementary figure 2**). Thus, we concluded that off-targeting events were not of major concern in either isogenic line.

3.2. A more efficient way for knock-in gene targeting

We had previously attempted to use the conventional ssODN and RNP approach to repair point mutations in iPS cells. Because Cas9 editing efficiency was tightly correlated with the mutation site, we had to choose a sgRNA in the same exon, ideally as close as possible to the mutation site. This largely limited the choices of sgRNA. To identify

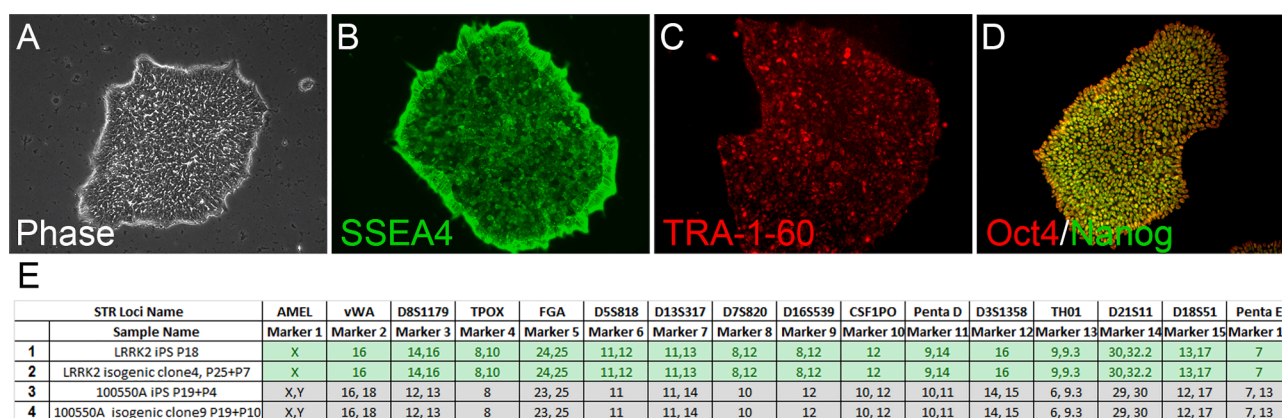


Fig. 4. Characterization of restored isogenic cells. A, an LRRK2 isogenic colony shows smooth boundary, the typical morphology of a colony of undifferentiated cells. B,C, The cells in the colony are all positive for stem cell surface markers SSEA4 and TRA-1-60 when stained with the marker for each in live-cell imaging. D, All cells in the colony are also positive for stem cell nuclear markers Oct4 and Nanog when stained after fixation. All four stem cell markers were uniformly expressed in individual cells. E, The STR analysis shows that the marker patterns of LRRK2 isogenic clone 4 perfectly match with those of the parental LRRK2 line, while the marker patterns of isogenic clone 9 match with those of its parent 100550A line.

Table 1

comparison of the gene editing efficiencies (AARS1 gene) from traditional method and new strategy described in this manuscript.

	Traditional ssODN and RNP method	New donor vector and RNP method
Single cell clone tested No.	96	47(after CRE transfected)
Edited clone No. (PCR analysis)	4	15
Editing rate	4.2%	31.9%
Confirmed clones (sequencing or ddPCR)	0	2 out of 5 (after CRE vector transfected)
Success rate	0%	40%

The AARS1 mutation c.986G > A is heterozygous. The lower success rate than editing rate may be caused by CRISPR-Cas9 mediated gene targeting happened on the wild type allele, leaving mutated allele unchanged.

positive clones, we needed to introduce a silent mutation to construct a restriction enzyme site for the subsequent characterization when designing ssODN. In addition, genotyping hundreds of clones and performing restriction enzyme reactions was tedious and time-consuming. Despite having screened hundreds of clones, we found only a couple of positive clones, all of which contained undesired additions or deletions at the Cas9 cleavage site causing frameshifting of the reading frame. We have thus developed this new strategy, which yields a much higher positivity rate due to the ability to engage an antibiotic selection (Table 1). In addition, the flexibility of the Cas9 cleavage location also provides a greater chance of selecting a more efficient sgRNA. Because Cas9 enzyme cleavage in this method occurs in an intron, we were able to avoid potential NHEJ events interfering with exon expression. The sequencing result from LRRK2 isogenic clones also showed that the corrected exon has no frameshifting or other mutations. We note that this same strategy is successful with the two targeted genes, even though LRRK2 isogenic lines have both alleles targeted while 100550A isogenic lines have only one allele targeted. Targeting efficiency may be further improved by varying the concentration of puromycin, the starting time of puromycin treatment after transfection, and the sequence context for designing sgRNA cleavage. It is mere coincidence that mutations in our two patient-derived iPSC lines are both G > A. Since the correction is introduced on a homologous arm, we predict that this strategy should work in other point mutations or frameshifting. It is noteworthy that our two mutations happen to be relatively close to a nearby intron making it possible to find a specific and efficient gRNA for Cas9 cutting. For mutations of intronless coding regions or mutations where no nearby introns can be found, our method will probably not work. In conclusion,

compared to the traditional approach for knock-in gene targeting, this new strategy provides the possibility of antibiotic selection, flexibility of sgRNA design, avoids possible NHEJ events and therefore has the potential to advance the use of iPSC lines as disease models.

CRedit authorship contribution statement

Jingli Cai: Conceptualization, Methodology, Investigation, Writing - original draft. **Elizabeth Kropf:** Resources, Investigation. **Ya-Ming Hou:** Conceptualization, Writing - review & editing, Funding acquisition. **Lorraine Iacovitti:** Conceptualization, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2021.102332>.

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