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Jingli Cai
*Thomas Jefferson University*

Elizabeth Kropf
*Thomas Jefferson University*

Ya-Ming Hou
*Thomas Jefferson University*

Lorraine Iacovitti
*Thomas Jefferson University*

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

Jingli Cai\textsuperscript{a}, Elizabeth Kroft\textsuperscript{a}, Ya-Ming Hou\textsuperscript{b}, Lorraine Iacovitti\textsuperscript{b, \textast}}

\textsuperscript{a} Department of Neuroscience Vickie & Jack Farrell Institute for Neuroscience, Sidney Kimmel Medical College, Thomas Jefferson University, 900 Walnut Street, JHN Suite 461, Philadelphia, PA 19107, USA
\textsuperscript{b} Department of Biochemistry and Molecular Biology, Sidney Kimmel Medical College, Thomas Jefferson University, 233 South 10th Street, BLSB Suite 220, Philadelphia, PA 19107, USA

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\textbf{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\textsuperscript{R} cassette. After selection with puromycin, positive clones were identified and further transfected with a CRE vector to remove the PGK-Puro\textsuperscript{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 \textit{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 \textit{Ran} \textit{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 \textit{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 \textit{et al.}, 2016). Also using asymmetric donor DNA can increase the HDR efficiency (Richardson \textit{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 \textit{et al.}, 2015; Harmsen \textit{et al.}, 2018; Paquet \textit{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 \textit{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 \textit{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 \textit{et al.}, 2016). Current base editing techniques only allow base substitutions of C\textsuperscript{G} to T\textsuperscript{A} that is mediated by cytosine base editors.
neology 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 drugs in therapies. In this study, the use of control isogenic cell lines derived from the same patient iPSCs 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 marked the sequences of the vector.

2.3. Gene targeting in hiPSCs

A million of LRRK2 or 100550A iPSCs 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′-GAAGCTACTACGTGGAGGTAC-3′, AARS1: 5′-GGGCGTATCGGA-CAGCTGCG-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 iPSCs or 100550A iPSCs 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-P10 were used for confirmation of the 5′ insertion. Primer set Puro-pA-P3-2 and LRRK2-P4, AARS1-P4 and Puro-pA-R-P10 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    | ATTCGGTCTGCTGTTGCTCT |
| Puro-pA-P3-2 | GCGACCCTCTTCCACATCC |
| LRRK2-P4    | AGCAAAATGATGATAGCCAC |
| LRRK2-P5    | TAAAGGACAAAAGTGACACAG |
| LRRK2-P6    | ATCTGAAGGTGTTGCTATCC |
| Puro-pA-R-P10| GCAGTTAATCTTCGAGTC |
| AARS1-P1    | AGCTAGCTAGTTGATGAGT |
| AARS1-P4    | CAGAGAATGAGAACGGCCAC |
| AARS1-P5    | TGGACGGGTAATGGAACAC |
| AARS1-P6    | TTCTAGGCGGTTACAGGAGC |

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 extracted from iPSCs 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 | caagcttggtaccgagctcgGCTCTGTTCCACAATGTGGTGAAGTCGTAAGTTATG | puro-pA-P3-2 |
| LRRK2-R arm | TTATGGGCGCCTGGAGGATTGATACAGCACAG | GCAGAGGTGTTGCTATCC |
| AARS1-L arm | caagcttggtaccgagctcgGATTGAGGATTGATAACATTGCACAG | CTATAGGATGAGTATCAG |
| AARS1-R arm | cttcgagttgatccagtGATTGGGTATGTTGCAAGTTTACAG | TTCTAGGCGGTTACAGGAGC |

2.1. Cell culture

LRRK2 (ND40018*C) iPSC cell line was obtained from NINDS human genetics DNA and cell line repository. Fibroblasts harboring the R329H mutation were isolated from an anonymous patient in Australia from the most common variant of LRRK2 (West et al., 2005). This c.986G•C that is mediated by adenine base editors (ABEs). Consequently, the speed of base editing can be slow.
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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: CCGTGATGGAATCTTGTTGCT; ANXA9-R: CATCTTATGTCATGGCGG). The corresponding genomic region of PTX4 was amplified by the primers from both isogenic and parental 100550A lines (PTX4-F2: ATCCACCTTGGTGATGGGA; 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...

Fig. 1. A stress-free strategy of correcting point mutations in patient iPSC cells. 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).
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® 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.
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® 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 the 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® 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
Table 1

| STR Loci Name | AMEL | vWA | DPB1*070 | TPOX | FGA | D5S818 |
|---------------|------|-----|----------|------|-----|--------|
| 1             | LRRK2 isogenic clone 1, P25*97 | X, Y | 15, 14.5 | 8.1 | 24, 23 | 12, 11 |
| 2             | LRRK2 isogenic clone 2, P25*97 | X, Y | 15, 14.5 | 8.1 | 24, 23 | 12, 11 |
| 3             | 100550A isogenic clone 9, P19*94 | X, Y | 15, 14.5 | 8.1 | 24, 23 | 12, 11 |
| 4             | 100550A isogenic clone 1, P25*97 | X, Y | 15, 14.5 | 8.1 | 24, 23 | 12, 11 |

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.

In conclusion, contractions 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 iPS 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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