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CRISPR/Cas9-AAV Mediated Knock-in at *NRL* Locus in Human Embryonic Stem Cells

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Clustered interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-mediated genome engineering technologies are sparking a new revolution in biological research. This technology efficiently induces DNA double strand breaks at the targeted genomic sequence and results in indel mutations by the error-prone process of nonhomologous end joining DNA repair or homologous recombination with a DNA repair template. The efficiency of genome editing with CRISPR/Cas9 alone in human embryonic stem cells is still low. Gene targeting with adeno-associated virus (AAV) vectors has been demonstrated in multiple human cell types with maximal targeting frequencies without engineered nucleases. However, whether CRISPR/Cas9-mediated double strand breaks and AAV based donor DNA mediated homologous recombination approaches could be combined to create a novel CRISPR/Cas9-AAV genetic tool for highly specific gene editing is not clear. Here we demonstrate that using CRISPR/Cas9-AAV, we could successfully knock-in a *DsRed* reporter gene at the basic motifleucine zipper transcription factor (*NRL*) locus in human embryonic stem cells. For the first time, this study provides the proof of principle that these two technologies can be used together. CRISPR/Cas9-AAV, a new genome editing tool, offers a platform for the manipulation of human genome.
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Subject Category: Gene addition, deletion and modification

Introduction

The ability to perform precision genome engineering has a broad range of research and medical applications, for example in food production, energy solutions, drug discoveries and medicine.[1,2] Site-directed genome editing tools include zinc finger nucleases and TAL effector nucleases, which are based on the principles of DNA-protein recognition.[3] Both zinc finger nucleases and TAL effector nucleases are chimeric proteins fusing a DNA-recognizing domain and the FokI nuclease catalytic domain. After heterodimerization of FokI, DNA double strand breaks at a targeted genomic locus will be generated. However, challenges in its design, synthesis, and validation have prevented widespread adoption of these engineered nucleases for routine use.[4,5] Clustered interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system has emerged as relatively simple and efficient genome editing method and is revolutionizing genomic engineering by equipping scientists with the ability to precisely modify the DNA of essentially any organism.[2,6]

CRISPR/Cas9 technology originates from Type 2 CRISPR/Cas systems, which provide bacteria with adaptive immunity to viruses and plasmids. The Cas9 is a nuclease that uses a guide sequence within an RNA duplex, tracrRNA:crRNA, to form base pairs with DNA target sequences, enabling Cas9 to introduce a site-specific double-strand breaks.[2] Double strand breaks in a targeted genomic locus can be repaired via nonhomologous end joining, or homologous recombination (HR). In HR, oligonucleotides are commonly used as donors.[7]

Genome engineering with adeno-associated virus (AAV) vectors has been demonstrated in multiple human cell types, with targeting frequencies ranging from 10^{-5} to 10^{-2} per infected cell.[8–11] These targeting frequencies are 1–4 logs higher than those obtained by conventional transfection or electroporation approaches. Through the use of AAV a wide variety of different types of mutations can be introduced into chromosomal loci with high fidelity and without genotoxicity. We hypothesized that if we introduce double strand breaks with CRISPR/Cas9 and then use AAV-based donor DNA-mediated homologous recombination, we could create a novel CRISPR/Cas9-AAV genetic tool for highly specific gene editing. Here we sought to study the use of CRISPR/Cas9-AAV in the manipulation of human genome.

Human embryonic stem cells (hESCs) can undergo unlimited self-renewal and retain pluripotency to differentiate into all cell types in the body, including photoreceptors.[12,13] Degeneration of photoreceptors in the retina is a common cause of blindness. Age related macular degeneration, in which cone photoreceptors in the central retina degenerate, is the leading cause of blindness in the elderly, while many inherited

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diseases of the retina result in rod photoreceptor degeneration.[14,15] There are currently no effective treatments available to prevent the loss of photoreceptors in most of these disorders. It has been reported that photoreceptor transplantation can restore vision after retinal degeneration in animal models, but cell replacement strategies will require a source of new retinal cells.[13,16] *NRL* is one of the key transcription factors that determine rod photoreceptor cell fate.[17]Theoretically, if we edit the *NRL* gene and knock-in a fluorescent reporter gene (*i.e.*, *DsRed*), it will be useful for the direct differentiation of hESCs to a retinal cell fate. In another word, *NRL DsRed* knock-in cells could be facilitated to track the direct differentiation process. Specially, when *NRL* gene turn on, *DsRed* gene will be expressed under the endogenous *NRL* promoter. Therefore, here we sought to use CRISPR/Cas9-AAV to manipulate *NRL* locus in hESCs.

Results

Optimization of hESC transfection conditions

Before we performed CRISPR/Cas9-mediated DNA cleavage with plasmids, we sought to optimize hESC transfection conditions. Because enzymatic specificity and activity are often highly depending on reaction conditions, for example, high enzyme concentrations may increase off-target activity.[18] In order to easily measure transfection efficiency, the plasmid pSin-eGFP was selected. The EF1a promoter in pSin-eGFP leads to robust promoter activity in hESCs.[19] Transfection efficiency and corresponding survival rate of cells were observed using a fluorescence microscope (**Supplementary Figure S1**). Notably, higher plasmid amounts reduced cell survival (**Supplementary Figure S1 B-a,C-a, D-a,B-b,C-b, and D-b**). With 1.25 µg plasmid DNA and a cell density of 5.0×10^5 cells/well in 12-well plates, we were able to achieve reliable and robust transfection with relative low levels of cell death. The heatmap (**Supplementary Figure S1a**) shows the relationship between relative pSin-eGFP transfection efficiency and cell survival rate.

CRISPR/Cas9-AAV mediated homologous targeting in hESCs

As illustrated in **Figure 1b**, we designed and assembled an *NRL* knock-in vector, which includes two homologous arms specific to the human *NRL* gene, a reporter gene positioned with the endogenous ATG site and one antibiotic expression cassette (PGK-NEO). Before infection, hESCs (H9) were treated with dispase to obtain a cell, suspension, thus each colony grown from single cell represents a single individual colony. To improve single-cell survival, 10 µmol/l Rho-associated kinase (ROCK) inhibitor Y-27632 was added 2 hours before treatment and post-treatment for 24 hours.[20] On day 0, 1.0×10^6 H9 cells/well were seeded in six-well plates. To minimize off-target effects, a double nickase of SpCas9 (pX335) strategy was adopted and the corresponding double strand breaks were introduced.[21] Specifically, sgRNA1 and sgRNA2 were designed to target the human *NRL* locus (**Figure 1a**). AAV vectors have been shown to mediate gene targeting at high frequencies.[8,22,23] Therefore, AAV2 vector, containing *NRL* homologous arms (5′ and 3′) and DsRed-PGK-NEO, was assembled (**Figure 1b**). We codelivered CRISPR/Cas9 with sgRNAs 1 and 2 (**Supplementary Table S2**), which would trigger specific genome cleavage in human ESCs at

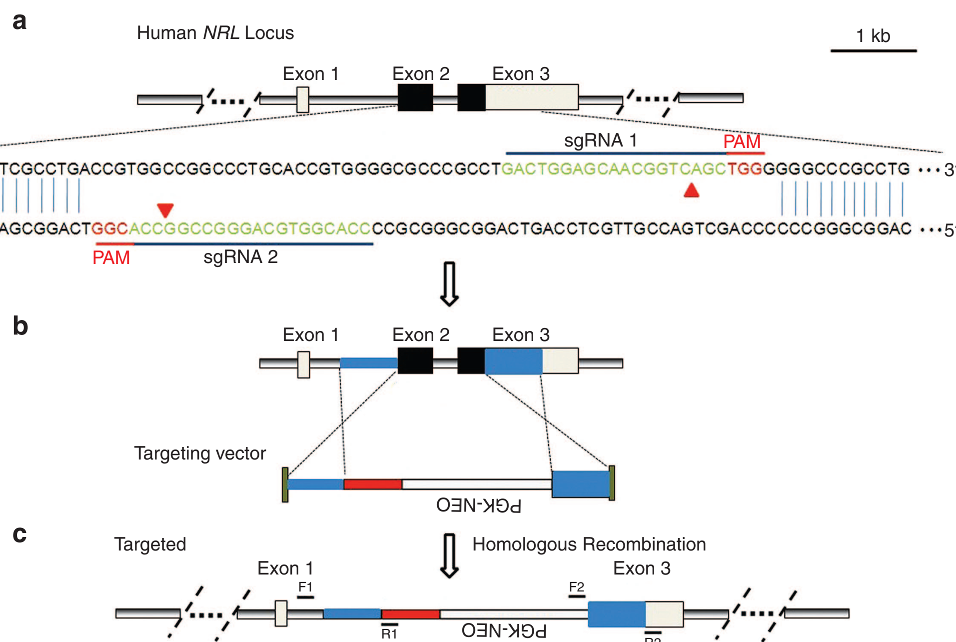

Figure 1 Schematic representation of CRISPR/Cas9-AAV at human *NRL* locus. (**a**) Schematic of the human *NRL* locus. sgRNAs and PAMs are labeled with gray line and red line, respectively. (**b**) Schematic representation of the vector for gene targeting. Blank boxes represent the coding sequence of *NRL* gene. Blue boxes represent the left and right homologous arms. Red box represents *DsRed* gene, the white box represents the coding region of the neo gene (*NEO*) and PGK promoter. (**c**) Targeted allele with homologous recombination. One of primers F1/R1 and F2/R2 locates outside of the homologous arms, respectively. PCR products will be obtained (824 and 823 base pairs with F1/R1 and F2/R2, respectively) with templates of genomic DNA from cells with homologous recombination. AAV, adeno-associated virus; CRISPR, Clustered interspaced short palindromic repeats; Cas9, CRISPR associated protein 9; PCR, polymerase chain reaction.

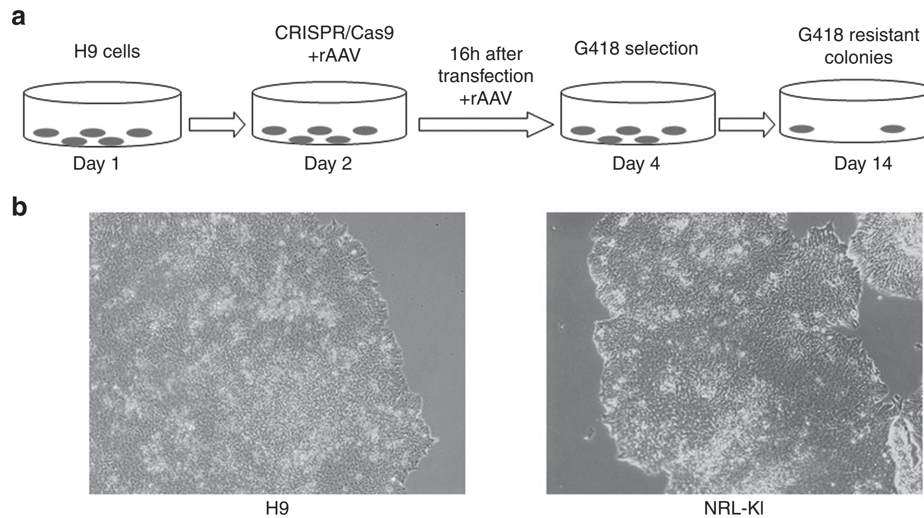

Figure 2 Generation of H9 *NRL* knock-in. (**a**) Schematic of protocol for *NRL*-KI in H9. (**b**) Bright-field phase images of the H9 cells (Parental cells, P) and *NRL* knock-in cells.

NRL locus, and infected the corresponding hESCs with rAAV. Thus, Exon 2 in one of *NRL* alleles would contain a knock-in DsRed-PGK-NEO through homology-directed repair (**Figure 1c**). After 16 hours it, fresh medium and rAAV was added again. On day 4, G418 was added. At day 14, G418 resistant (G418r) colonies were picked (**Figure 2a**).

To identify positive colonies, we designed primers (F1/R1 and F2/R2) and screened them with gene specific PCR (**Figure 1c**). Because one primer (F1 or R2) anneals to sequences located outside of the homologous arms, all the positive colonies screened with PCR should be homologous recombinant Ds-Red knock-in. The amplification products (5′ and 3′) were 824bp, 823bp, respectively (**Figure 3a**). Colony 2 was identified as positive by PCR screening. As 5′ PCR products have an EcoR I site, we digested the corresponding PCR fragments with EcoR I, which theoretically leads to differential patterns of bands (546bp and 274bp) on agarose gels. As we expected, the *NRL*-knock-in candidate had two fragments with the desired size, compared with the wild-type with fragment of 824bp (**Figure 3b**, Colony 2). Not surprisingly, random integration of the gene targeting vector was also observed (Colony 1). Taken together, these results showed that *NRL* knock-in hESCs can be obtained using the CRISPR/Cas9-AAV system.

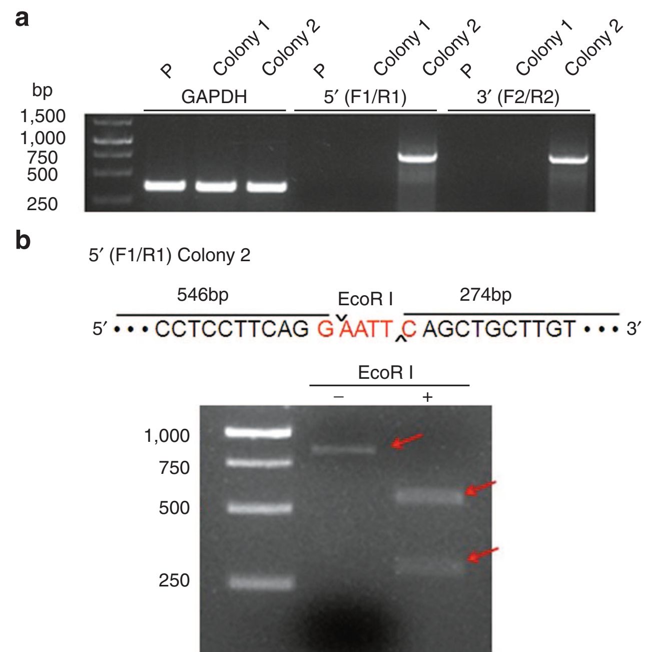

Figure 3 Polymerase chain reaction (PCR) analysis and confirmation of *NRL* knock-in. (**a**) PCR analysis identified colony 2 has *DsRed* gene knock-in at *NRL* locus. Location of F1/R1 and F2/R2 are illustrated in **Figure 1c**. (**b**) 5′ terminal PCR (with primers F1R1) products were digested with restriction enzyme EcoRI. P, parental cells.

Characteristics of CRISPR/Cas9-AAV edited hESCs

Next, we examined the morphology and proliferation characteristics of the knock-in colony. No notable change has been observed between the parental and knock-in hESCs (**Figure 2b**). Then we asked whether the knock-in H9 colony could still maintain the characteristics of hESCs.[24] Reverse transcription PCR (RT-PCR) showed that the hESC specific expresstion genes *SOX*2 (SRY (sex determining region Y)-box 2), *OCT4* (Octamer-binding transcription factor-4), and *NANOG* were robustly expressed in parental H9 cells and the knock-in colony (**Figure 4a**). Immunostaining for OCT4 confirmed that the knock-in colony had the same *OCT4* gene expression pattern as parental H9 cells (**Figure 4b**). Together, our data shows that after CRISPR/Cas9-AAV mediated gene editing, the knock-in hESCs has the same gene expression pattern of the specific stem cell markers both at the mRNA and protein level, Also, no clear differences were observed with the passage of these cells (**Figure 4a**).

Off-target analysis by whole exome sequencing

To address the potential off-target effects of CRISPR/Cas9-AAV, we performed whole exome sequencing. Genomic DNA was isolated from the parental and knock-in hESCs with the

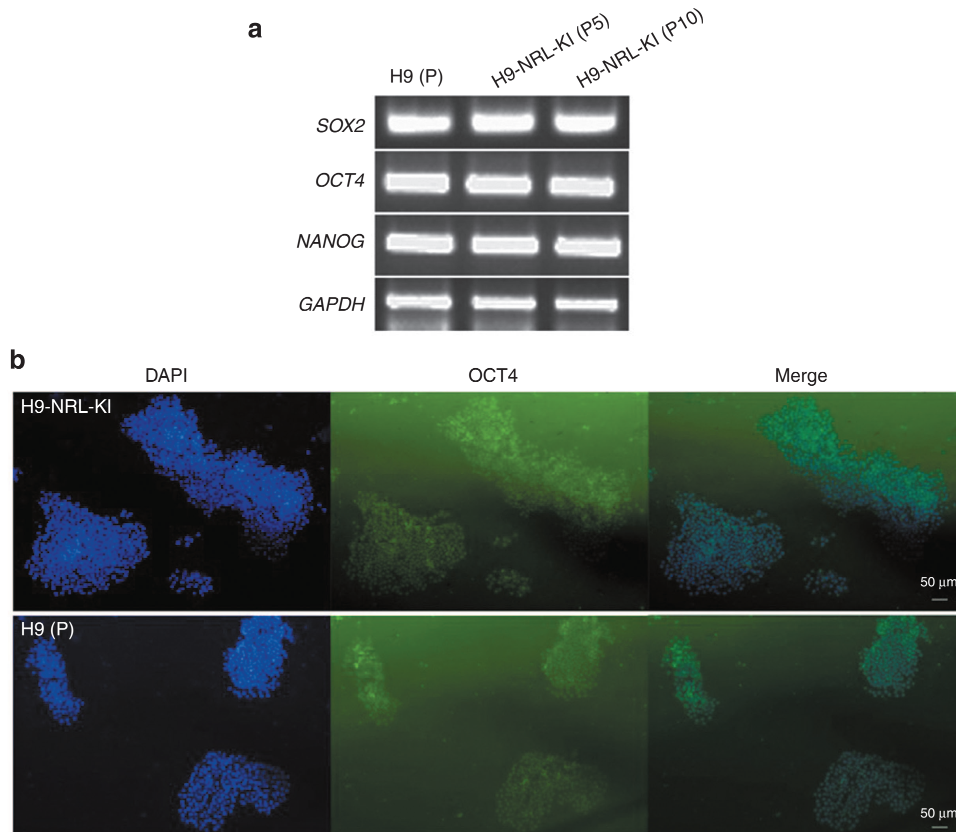

Figure 4 Characterization of *NRL* knock-in colony. (**a**) Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed the expression of *SOX*2, *OCT4*, and *NANOG* in knock-in colony with different passages (5 and 10). (**b**) OCT4 immunostaining analysis of H9 and *NRL* knock-in colony at passage 10. P, parental cells.

same passage. The whole exome libraries were constructed and then sequenced on an Illumina Solexa HiSeq 2000 sequencer. Whole exon sequencing analysis identified 41 genes with high confidence mutations, including six indels and 35 SNVs (**Supplementary Table S1**). To confirm these mutations, condidate deleterious mutation from 9 genes (*MAP3K1*, *RDH12*, *TRAK1*, *ATXN1*, *FAM47A*, *TNFRSF10C*, *OR4Q3*, *ZCCHC2*, and *RS1*) (**Supplementary Table S3**) were selected for confirmation. Primers (**Supplementary Table S2**) flanking the potential off-target sites were used to amplify the fragments harboring the potential mutations. The results of DNA sequencing showed that the 6 indel mutations identified by exome sequencing in knock-in cells were artifacts (**Supplementary Figure S2** and **Supplementary Table S3**). While, one (*ZCCHC2*, exon13, c.2711C>A, p. A904E) of three SNV mutations (*OR4Q3*, *ZCCHC2*, and *RS1*) was present (**Supplementary Figure S3; Supplementary Figure S4a** and **Supplementary Table S3**). To determine whether the *ZCCHC2* mutation is linked to sgRNA1 and sgRNA2, we searched for corresponding potential homologous sequences in a 500bp window around the mutation, this revealed that there was no homology in this region. Multiple-sequence alignment in *ZCCHC2* from different species revealed that these mutations were located within a highly conserved region (**Supplementary Figure S4b**). While, we can't exclude *ZCCHC2* (p. A904E) is spontaneous mutation. These results demonstrated that CRISPR/Cas9-AAV-mediated gene editing has very few off-targets effects.

Discussion

Human pluripotent stem cells, including hESCs and human induced pluripotent stem cells, hold great promise for cell therapy.[25] However, efficient differentiation of these cells into some specific subtypes remains challenging.[12,26] Generation of a specific reporter cell line is highly desirable for the direction of differentiation. However, manipulation of the human pluripotent stem cell genome has been technically challenging.[27] The efficiency of existing gene targeting approaches to create reporter cell lines is very low.[27,28] Gene targeting with AAV vectors has been demonstrated in hESCs with maximal targeting frequencies without engineered nucleases. However, so far, methods to edit silent loci are not efficient (all published studies are on active loci). Recently, several groups have demonstrated the use of CRISPR/Cas9 to modify the human pluripotent stem cell genome allowing precise temporal control of gene expression and elucidation of gene functions in human pluripotent stem cells.[29] For example, it has been shown that *NEUROG3* is essential for human endocrine pancreas development by generation of *NEUROG3*$^{-/-}$ hESC lines.[30] To dissect the function of *ELABELA,* the evolutionarily conserved 54-amino acid hormone

required for heart development in hESCs, CRISPR/Cas9 was used and showed that *ELABELA* promotes the growth and pluripotency of hESCs.[31] And high-fidelity CRISPR/Cas9 nucleases[32,33] have been discovered that would enhance the usage.

Photoreceptor derivation from hESCs and human induced pluripotent stem cells is possible.[34–36] *NRL* is a photoreceptor cell fate determinant,[37] and therefore CRISPR/Cas9 mediated knock-in of a fluorescent reporter gene in hESCs at *NRL* locus will be helpful for tracking the process of photoreceptor differentiation. Further studies of photoreceptor differentiation will be performed to taking advantage of the present reporter cell line.

Several types of DNA have been used as donors for homologous recombination (HR, or homology directed repair), including circular/linearized plasmids, PCR products and single-stranded oligonucleotides. The integration of a CRISPR/Cas9 mediated circular plasmid donor Drosophila *DSH3PX1* has been reported.[38] The circular plasmid has been chosen as a donor template for CRISPR/Cas9 mediated genome editing in human iPSC lines.[39] With linearized plasmid as the donor vector, homologous recombination was successfully achieved in 5% of human ESCs by CRISPR/Cas9 mediated homology directed repair.[39] In this study, 50% targeting efficiency has been obtained with CRISPR/Cas9-AAV at *NRL* locus in human ESCs. In another approach, PCR-product based homologous recombination was achieved with CRISPR/cas9 in *Drosophila* cells.[41] Single-stranded oligonucleotides have also been used in HR with CRISPR/cas9 cleavage of the zebrafish genome.[42] Here we provided the proof of principle that CRISPR/Cas9-AAV, a new genome editing tool, offers a platform for the manipulation of human genome. Additional advantages of this tool includes, as the linearized single strand DNA genome of AAV, it can't be selected as substrate for CRISPR/Cas9 cleavage. Thus, compared with double stands DNA HR template, AAV will be waived for CRISPR/Cas9 cleavage and then delivered as DNA HR template. Compared with single-stranded oligonucleotides as homology directed repair template, AAV has capacity of about 4,300 nt and delivers as virus by different serotypes to achieve robust infection with different cell types.

Although the canonical PAM for SpCas9 is NGG, previous studies (including ours) demonstrated active off-target sites with NGA, and NAG PAM sequences have been identified.[43,44] Mismatches as well as base insertions or deletions that form bulges between the target DNA strand and guide RNA sequences may also be tolerated. To minimize off-target effects, the double nickase SpCas9 strategy was adopted in the present study and corresponding double strand breaks were introduced. Specifically, a pair of offset sgRNAs (sgRNA1 and sgRNA2) positioned on the human *NRL* locus were designed. Other strategies has been reported, including an sgRNA-guided dCas9 fused to the FokI nuclease where two fused dCas9-FokI monomers can simultaneously bind target sites; and shorter sgRNAs truncated by two or three nucleotides at the distal end relative to the PAM that can be used with the double nicking strategy to further reduce off-target activity. Recently, novel high-fidelity Cas9s have been reported, which may increase the fidelity of CRISPR/Cas9 gene editing.[32,33]

Whole-genome sequencing of Cas9-edited human pluripotent stem cells can lead to the identification of low frequency yet potentially deleterious off-target events.[45] Deep sequencing has confirmed a high rate of mutagenesis at targeted loci, with a small subset of off-target sites exhibiting indels by genome-scale CRISPR/Cas9 knockout screening in human cells.[46] In this study, we identified several DNA variants after *NRL* gene editing and we do not know the reasons underlying this. Very few papers have been published about the off-targets issue of gene targeting with AAV. In this study, whether the off-target *ZCCHC2* mutation comes from AAV mediated gene targeting or caused by CRISPR/Cas9 mediated DNA cleavage or by spontaneous mutation is not known. Additional studies should been undertaken to analyze the potential genome modifications induced by off-target effects.

Materials and methods

Vector construction. The *NRL* donor vector was assembled with a 5′ homologous arm (675 bp), DsRed-PGK-NEO and 3′ homologous arm (655 bp), as illustrated in **Figure 1b**. pX335-sgRNAs targeted to *NRL* locus were constructed as described online (http://www.genome-engineering.org/crispr/).[43] The oligonucleotide sequences are summarized in **Supplementary Table S2**.

Cell culture. Human H9 cells were cultured on plates coated with Matrigel (Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-Free, Corning, Tewksbury, MA) with mTeSR medium (Stemcell Technologies, Vancouver, BC) supplemented with 5× supplement. Y-27632 dihydrochloride (Sigma-Aldrich) was added during 1–2 days after thawing. After gene editing, 50 µg/ml G418 was added to the medium for the selection of resistant colonies.

Cell transfection and infection. The hESCs were digested by dispase (Roche, Mannheim, Germany) and seeded in 12-well plates at a density of $1.0/2.5/5.0 \times 10^5$ cells/well and 0.5/1.25/2.5 µg plasmid were transfected respectively, as described in the manufacturer's protocol (Roche). The relative efficiency of pSin-eGPF transfection was measured under a fluorescent microscope.

CRISPR/Cas9-AAV mediated knock-in at the human *NRL* locus was performed. Specifically, 1.0×10^6 H9 cells were seeded in one well of a six-well plate and cotransfected with 2.5 µg CRISPR/Cas9 plasmids (sgRNA 1 and sgRNA 2). AAV2 was used as donor vector for homologous recombination at a multiplicity of infection of 10,000. Post-transfection of 16 hours, fresh medium was added and the same amount of rAAV2 was added twice. At day 14, G418 resistant colonies were obtained.

Knock-in screening. Genomic DNA from G418 resistant colonies was extracted as previously described.[43] Polymerase chain reaction (PCR) was used to screen the candidate knock-ins, using one primer (F1 or R2) annealing to sequences located outside of the homologous arms, and thus only homologous recombinant (knock-in) had specific

amplification. The amplification products (F1/R1 and F2/R2) were 824 and 823 bp, respectively. Restriction fragment length polymorphism was used for the confirmation of knock-in.

Off-target analysis. Off-target alterations were analyzed by whole exome sequencing. Sanger sequencing were used to confirm the results of whole exome sequencing. ABI genomic DNA from H9 and H9-NRL-knockin cells were used as the templates for the amplification. The PCR products were directly sequenced using one of the primers (**Supplementary Table S2**).

Reverse transcription-PCR. Total mRNAs from H9, H9-NRL-KI (p5 and p10, two different passages) cells were extracted using TriZol (Invitrogen, Carlsbad, CA). cDNA was reverse transcribed by M-MLV Reverse Transcriptase (Promega, Madison, WI). Primer sequences for *SOX2*, *OCT4*, *NANOG* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) can be found in the **Supplementary Table S2**.

Immunostaining. Cells were fixed with 4% paraformaldehyde and immunolabeled as described previously.[47] The primary antibody Oct4 (R&D Minneapolis, MN) was used for staining for *OCT4* gene expression.

Supplementary material

Figure S1. Optimization transfection condition of hESCs using pSin-eGFP plasmid.
Figure S2. DNA sequencing analysis of indel mutations.
Figure S3. DNA sequencing analysis of *RS1* and *OR4Q3* mutations.
Figure S4. DNA sequencing analysis of *ZCCHC2* mutation.
Table S1. Summary of whole exon sequencing with H9 and NRL knock-in cells.
Table S2. Lists of oligo sequences used in this study.
Table S3. Summary of off-targets analysis.

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