Generation of a homozygous LRP2 knockout human embryonic stem cell line (FDCHDPe010-A-56) by CRISPR/Cas9 system

Jie You\textsuperscript{a,b,c}, Yun Cheng\textsuperscript{a,b,c}, Xian-Jie Yang\textsuperscript{d}, Ling Chen\textsuperscript{a,b,c,*}

\textsuperscript{a} Department of Ophthalmology & Vision Science, Eye & ENT Hospital, Shanghai Medical School, Fudan University, Shanghai, China

\textsuperscript{b} Key NHC Key Laboratory of Myopia (Fudan University), Laboratory of Myopia, Chinese Academy of Medical Sciences, China

\textsuperscript{c} Shanghai Key Laboratory of Visual Impairment and Restoration, Eye & ENT Hospital, Shanghai Medical School, Fudan University, Shanghai, China

\textsuperscript{d} Jules Stein Eye Institute, University of California Los Angeles, CA, USA

Abstract

LRP2 is mainly expressed in the cell membrane of epithelia, maintaining normal endocytosis of nutrients from the extracellular microenvironment and mediating growth factor signals. The deficiency of LRP2 can result in abnormal lysosomal and mitochondrial function as well as insufficient resistance to oxidative stress. LRP2-KO animals show enlarged eyes and malfunction of the retinal pigment epithelium (RPE). We were able to generate an LRP2-KO human embryonic stem (ES) cell line using CRISPR/Cas9 gene editing and differentiate the mutant ES cells into RPE cells. Thus, this LRP2-KO human ES line will facilitate studying cellular mechanisms of eye disease due to LRP2 deficiency.

2. Resource utility

Low-density lipoprotein receptor-related protein 2 (LRP2) is a cell surface protein mainly expressed in the apical surface of epithelia. LRP2 has been shown to be a multifunctional receptor, playing roles in maintaining normal endocytosis of nutrients and other substances, as well as mediating growth factor binding. Deficiencies of LRP2 can result in perturbed membrane trafficking and dysfunctional lysosomes and mitochondria (Marzolo MP, 2011). Humans with recessive LRP2 mutations develop Donnai-Barrow syndrome with craniofacial anomalies including ocular hypertelorism, forebrain defects, and mild holoprosencephaly (Christ et al., 2012; Rosenfeld et al., 2010). LRP2 knockout mice show an enlarged ocular...
appearance, and exhibit abnormal retinal pigment epithelium (RPE) endocytosis (Cases et al., 2017; Patel et al., 2007). To establish human disease models, we generated an LRP2-KO human embryonic stem cell (HESC) line using CRISPR/cas9 system. Differentiating LRP2-KO ES cells into RPE cells will further studies of LRP2 function in ocular development and diseases.

3. Resource details

To generate the LRP2 knock-out HESC line, we used a CRISPR/cas9 system based on Staphylococcus aureus Cas9 (SaCas9), which edits the genome with efficiencies similar to those of SpCas9, while being > 1 kb shorter (Ran et al., 2015). To improve the efficiency of screening for mutated clones, a DNA fragment containing the CMV promoter sequence, GFP sequence, and poly-A sequence was inserted into XhoI endonuclease sites of the px601 plasmid (Addgene #61591) by homologous recombination. A guide RNA was designed targeting the second exon of the common sequence of three transcripts (exon17 of transcripts 1 and 2; exon 3 of transcript 3) of LRP2 (Fig. 1A) by utilizing http://crispor.tefor.net/crispor.py. The synthesized oligo was inserted into the vector described above. Then, the plasmid was transfected into wild-type HESCs (H9 [Wi Cell Research Institute, Madison, WI, USA]) by lipofection. Forty-eight hours later, GFP positive cells were sorted by FACS and seeded as single cells in the presence of ROCK inhibitor.

Among all the expanded HESC clones, a single clone carrying a 2-bp deletion (Fig. 1B), as confirmed by Sanger sequencing, was chosen for further research. The deletion resulted in a frameshift mutation at amino acid No.96 (No.870 of transcripts 1,2; NO.107 of transcript 3) in the common sequences of the three transcripts, leading to a premature stop codon at amino acid No.117 (No.891 of transcripts 1,2; NO.128 of transcript 3; Fig. S1A), and truncation of the subsequent 3764 amino acid that contains critical domains, conserved sites, including the transmembrane domain (Fig. S1B). In addition, the CRISPR cut site in the LRP2 protein is predicted to be part of LDLR class B repeat (No.415–4304aa of transcript 1,2; No.1–3541aa of transcript3) and thus may disturb the formation of the predicted beta-propeller structure, which is critical for ligand release and recycling of the receptor (Davis et al., 1987; Springer, 1998).

The LRP2 gene-edited cell line showed morphologies typical of pluripotent stem cells (Fig. 1E). Immunostaining studies with the LRP2-KO HESC line showed the expression of several pluripotency markers, including OCT4, SOX2, and Nanog (Fig. 1C). Flow cytometry analysis further confirmed comparable percentages of OCT4 positive cells (>98%) in the LRP2-KO HESC line and the wild-type HESC line (Fig. 1D). Karyotype analysis demonstrated that the LRP2-KO cell line had a normal female karyotype (46, XX), with no gross chromosome structure abnormalities (Fig. 1I). Trilineage differentiation assays were conducted in vitro to confirm the expression of markers for ectoderm (PAX6), mesoderm (BRACHYURY), and endoderm (FOXA2) (Fig. 1F). Short tandem repeat (STR) analysis showed that the LRP2-KO HESC line matched the parental cell line of origin. These cells were free of mycoplasma contamination (Fig. 1J). Off-target analyses were conducted using primers from http://crispor.tefor.net/crispor.py, and Sanger sequencing confirmed that no predicted off-target sites were present in the LRP2 mutant HESC line. LRP2-KO HESC
cells were subsequently induced to differentiate into RPE, which displayed a polygonal morphology, produced pigment like human RPE, and expressed typical RPE markers ((ZO-1 in the cell membrane, MITF in the nucleus and RPE65 in the endoplasmic reticulum and plasma membrane) (Fig. 1G) (Buchholz et al., 2013; Foltz and Clegg, 2017). The absence of detectable levels of LRP2 protein in the differentiated LRP2-KO cells was confirmed by Western blot (Fig. 1H). The above pieces of information of the LRP2-KO hESCs were summarized in Resource Table and Table 1.

4. Materials and methods

4.1. Cell culture

Wild-type HESC and LRP2-KO HESC lines were cultured in mTeSR™1 (Stem Cell Technology) on Matrigel®-coated plates (Corning). When colonies reached 70–80% confluency, ReLeSR™ (Stem Cell Technology) was used to detach and dissociate large clones. Cells were passaged at a 1:3 ratio, and single cells were obtained using Accutase (Sigma-Aldrich) before plasmid transfections and FACS sorting. Post-FACS recovery medium was utilized to promote adherence of single cells (Peters et al., 2008).

4.2. Gene targeting

LRP2-sgRNA was designed, synthesized, and cloned into the vector described above. Lipofetamine3000 (Thermofisher Scientific) was then used to transfect 5ug of the engineered plasmid into 5×10⁵ HESCs. GFP positive cells were sorted through FACS and seeded as single cells until large enough for screening. Clones were manually picked and genomic DNA used for Sanger sequencing using primers listed in Table 2. Only clones that showed appropriate indels at the designed sgRNA targeting site were selected for further analyses.

4.3. Immunostaining

Passaged cells were seeded on a 24-well plate and cultured for 3–4 days. After 3 washes with PBS, cells were fixed in 4% paraformaldehyde for 10–15 min, permeabilized with 0.2% Triton X-100 for 15 min, and blocked in 4% bovine serum albumin for 30 min at room temperature. Cells were then incubated with primary antibodies at 4 °C overnight, followed by secondary antibodies for 1 h at room temperature. Both primary and secondary antibodies were diluted in the blocking medium. Cell nuclei were stained with DAPI (Abcam) for 5 min at room temperature. Images were taken by an inverted fluorescence microscope (Leica Microsystems, Germany). The antibodies used are listed in Table 2.

4.4. Flow cytometry analysis

Typically, 1×10⁶ cells were washed twice with PBS and fixed in 4% formaldehyde for 15 min at room temperature. Cell permeabilization was performed by slowly adding 100% cold methanol to pre-chilled cells to a final concentration of 90% methanol, and then incubating for an additional 10 min on ice. Cells were subsequently incubated with primary antibody for 1 h and then with the corresponding secondary antibody for 30 min at room temperature. The primary and secondary antibodies were diluted with 3% BSA. Cells were resuspended in 500ul pre-chilled PBS, detected with flow cytometry (Beckman coulter Inc, MoFlo
XDP), and analyzed for percentages of signal-positive cells among total cells by Summit5.2 software.

4.5. Differentiation of HESC and LRP2-KO HESC

Trilineage differentiation assays were carried out according to instructions of the Trilineage differentiation kit (Stem Cell Technology). Briefly, appropriate amounts of ES cells were seeded on Matrigel-coated plates. Then, cells were treated with lineage-specific differentiation medium respectively. About 5 or 7 days later, typical germ layer markers were detected by immunostaining. The antibodies used are listed in Table 2.

For directed differentiation of LRP2-KO HESC cells into RPE, a previously published protocol was used with mild modification (Foltz and Clegg, 2017) and cytokines were added step by step to induce stem cell transformation.

4.6. Western blot

Cells were washed twice with cold PBS, incubated with RIPA lysate containing 1% PMSF on ice for 15 min, and centrifuged at 12000 rpm for 15 min to obtain the supernatant. After SDS-PAGE electrophoresis (Tanon Science Inc, Shanghai), the lysates were transferred to 0.45um PVDF membranes and incubated with LRP2-specific polyclonal antibody (Proteintech Group) at 4°C overnight. Then incubated for 1 h at room temperature with secondary antibody, rinsed, and detected by chemiluminescence with HRP substrate (Millipore). The antibodies used are described in Table 2.

4.7. Karyotype analysis

Cells in their logarithmic growth phase were treated with 10ug colchicine and then incubated for 4 h at 37°C, 5% CO2. Single cells were obtained using Accutase. Standard cytogenetic procedures were performed by ZhenHe Bioscience Inc, Shanghai using the GTG-band method.

4.8. STR analysis

STR analysis was authenticated by iCell Bioscience Inc, Shanghai.

4.9. Off-target analysis

Potential off-target sites (POTs) were identified using the website service http://crispor.tefor.net/crispor.py to predict possible site-specific cleavage by CRISPR/Cas9. The PCR products of POTs were confirmed by Sanger sequencing. POT primers are listed in Table 2.

4.10. Mycoplasma test

Mycoplasma tests were performed using the EZ-PCR Mycoplasma Test Kit (Biological Industries, BI) following the manufacturer’s instructions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.
Funding

This study was funded by the National Natural Science Foundation of China (No.81870660) and Shanghai Science and Technology Foundation (18ZR1405900) to L. Chen.

References

Buchholz DE, Pennington BO, Croze RH, Hinman CR, Coffey PJ, Clegg DO, 2013. Rapid and efficient directed differentiation of human pluripotent stem cells into retinal pigmented epithelium. Stem Cells Transl. Med. 2, 384–393. [PubMed: 23599499]

Cases O, Obry A, Ben-Yacoub S, Augustin S, Joseph A, Toutirais G, Simonutti M, Christ A, Cosette P, Kozyraki R, 2017. Impaired vitreous composition and retinal pigment epithelium function in the FoxG1::LRP2 myopic mice. Biochim. Biophys. Acta, Mol. Basis Dis. 1863, 1242–1254. [PubMed: 28366874]

Christ A, Christa A, Kur E, Lioubinski O, Bachmann S, Willnow TE, Hammers A, 2012. LRP2 is an auxiliary SHH receptor required to condition the forebrain ventral midline for inductive signals. Dev Cell 22 (2), 268–278. [PubMed: 22340494]

Davis CG, G JL, Südhof TC, Anderson RG, Russell DW, Brown MS, 1987. Aciddependent ligand dissociation and recycling of LDL receptor mediated by growth factor homology region.pdf. Nature 760–765.

Foltz LP, Clegg DO, 2017. Rapid, directed differentiation of retinal pigment epithelial cells from human embryonic or induced pluripotent stem cells. J Vis Exp.

Marzolo MP FP, 2011. New insights into the roles of Megalin LRP2 and the regulation of its functional expression.pdf. Biol. Res. 89–105. [PubMed: 21720686]

Patel N, Hejkal T, Katz A, Margalit E, 2007. Ocular manifestations of donnai-barrow syndrome.pdf. J. Child Neurol. 22, 462–464. [PubMed: 17621530]

Peters DT, Cowan CA, Musunuru K, 2008. Genome Editing in Human Pluripotent Stem Cells. StemBook, Cambridge (MA).

Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F, 2015. In vivo genome editing using Staphylococcus aureus Cas9. Nature 520, 186–191. [PubMed: 25830891]

Rosenfeld JA, Ballif BC, Martin DM, Aylsworth AS, Bejjani BA, Torchia BS, Shaffer LG, 2010. Clinical characterization of individuals with deletions of genes in holoprosencephaly pathways by aCGH refines the phenotypic spectrum of HPE. Hum Genet 127 (4), 421–440. [PubMed: 20066439]

Springer TA, 1998. An extracellular β-propeller module predicted in lipoprotein and scavenger receptors, tyrosine kinases, epidermal growth factor precursor, and extracellular matrix components.pdf. J. Mol. Biol. 837–862. [PubMed: 9790844]
Fig. 1.
Characterization of LRP2 knockout human embryonic stem cell line (FDCHDPe010-A-56).
## Table 1

Characterization and validation.

| Classification | Test | Result | Data |
|----------------|------|--------|------|
| Morphology     | Photography | Normal | Fig. 1 panel E |
| Pluripotency status evidence for the described cell line | Qualitative analysis (i.e. Immunocytochemistry, western blotting) [mandatory] | Positive for OCT4, SOX2, NANOG | Fig. 1 panel C |
| Karyotype      | Karyotype (G-banding) and higher-resolution, array-based assays (KaryoStat, SNP, etc.) | Flow cytometry: Oct4 > 98% | Fig. 1 panel D |
| Genotyping for the desired genomic alteration/allelic status of the gene of interest | PCR across the edited site or targeted allele-specific PCR | PCR + sequencing: Homozygous 2-bp deletion | Fig. 1 panel B, H |
| Verification of the absence of random plasmid integration events | PCR/Southern | PCR detection: No plasmid backbones | Figure S1 panel C |
| Parental and modified cell line genetic identity evidence | STR analysis, microsatellite PCR (mPCR) or specific (mutant) allele seq | 9 loci tested; Available with the authors | DSS818, D13S117, D7S820, D16S539, vWA, Th01, AMEL, TPOX, CSF1PO, 100% matched; Available with the author |
| Mutagenesis / genetic modification outcome analysis | Sequencing (genomic DNA PCR or RT-PCR product) | PCR + sequencing: Homozygous 2-bp deletion | Fig. 1 panel B |
| Specific pathogen-free status | Mycoplasma | Negative | Fig. 1 panel J |
| Multilineage differentiation potential | Trilineage differentiation | Expressing three germ layers formation: Ectoderm (PAX6), Mesoderm (BRACHYURY) and Endoderm (POXA2) | Fig. 1 panel F |
| Donor screening (OPTIONAL) | HIV 1 + 2 Hepatitis B, Hepatitis C | N/A | N/A |
| Genotype - additional histocompatibility info (OPTIONAL) | Blood group genotyping | N/A | N/A |
| | HLA tissue typing | N/A | N/A |
### Table 2

#### Reagents details.

| Antibodies and stains used for immunocytochemistry/flow-cytometry | Antibody          | Dilution | Company Cat # and RRID                      |
|------------------------------------------------------------------|-------------------|----------|--------------------------------------------|
| Pluripotency Markers                                             | Rabbit anti-OCT4  | 1:100 for immunostaining 1:60 for flow cytometry | Abcam Cat# ab181557, RRID: AB_2687916 |
|                                                                  | Rabbit anti-SOX2  | 1:100    | Abcam Cat# a92494, RRID: AB_10585428       |
|                                                                  | Rabbit anti-NANOG | 1:100    | Abcam Cat# ab21624, RRID: AB_446437        |
| Differentiation Markers                                          | Rabbit anti-PAX6  | 1:100    | BioLegend Cat# PRB-278P, RRID: AB_291612   |
|                                                                  | Rabbit anti- BRACHYURY | 1:100 | Abcam Cat# ab209665, RRID: AB_2750925     |
|                                                                  | Rabbit anti-FOXA2 | 1:100    | Abcam Cat# ab108422, RRID: AB_11157157     |
| RPE Markers                                                      | Rabbit anti-ZO-1  | 1:100    | Thermo Fisher Scientific Cat# 402300, RRID: AB_2533456 |
|                                                                  | Mouse anti-MITF   | 1:100    | Abcam Cat# ab3201, RRID: AB_303601         |
|                                                                  | Mouse anti-RPE65  | 1:100    | Novus Biologicals Cat# NB100-35 RRID:     |
|                                                                  |                   |          | AB_10002148                               |
| Western Blot analysis                                            | Rabbit anti-LRP2  | 1:300    | Proteintech Cat# 9700-1-AP, RRID: N/A      |
| Secondary antibodies                                             | Alexa Fluor 594 AffiniPure Donkey Anti-Rabbit IgG (H + L) | 1:1000 | Yeasen Cat# 34212ES60, RRID: N/A        |
|                                                                  | AlexaFlour488 goat anti-rabbit IgG | 1:1000 | Thermo Fisher Scientific Cat# A-11008, RRID: AB_143165 |
|                                                                  | AlexaFlour555 goat anti-mouse IgG | 1:1000 | Thermo Fisher Scientific Cat# A-21422, RRID: AB_2535844 |
|                                                                  | Peroxidase-Conjugated Goat Anti-Rabbit IgG (H + L) | 15000 | Yeasen Cat# 33101ES60, RRID: N/A        |
| Nuclear stain                                                     | DAPI              | 1:100    | Abcam Cat# ab104139, RRID: N/A            |
| Site-specific nuclease Nuclease information                      | N/A               | N/A      |                                            |
| Delivery method                                                  | FACS              | FACS     |                                            |
| Selection/enrichment strategy                                    |                   |          |                                            |
| Primers and Oligonucleotides used in this study                  | Target            |          | Forward/Reverse primer (5′–3′)             |
| Targeted mutation analysis/sequencing                             | LRP2, 2nd Exon of common sequence of three transcripts | GCAGTATCGGAGAATCTCCTGTTT/ |
| gRNA oligonucleotide/crRNA sequence                              | LRP2              |          | GAGTTTCCACTAAATCTTGTTCATTGCAGC             |
| Potential random integration-detecting PCRs                      | PB1               |          | ATACGATGTCCAGATACGCT/ GGTGTTTCGTCCCTTCACAAG |
| Antibody | Dilution | Company Cat # and RRID |
|----------|----------|-----------------------|
| PB2      | CGTGTATCTCGCTACTGTTGG/CCAGTTTGGAACAAGAGTCCACTAT | |
| PB3      | CTCGAGGCGTTGACATGGAT/GTGGCACCGGATGTTAGCC | |
| Top off-target mutagenesis predicted site sequencing | | |
| POT1     | CTTTGCCTGGCCAAGATTC/TCTGTCAGGCATCATGCTGG | |
| POT2     | GTGGCATTCGCAATTCTGGC/CTGTTCACACCAGACCTGAG | |
| POT3     | CAGATGTGTCAGAGCCCTCGATGAAACTCTGGGCAAAAG | |
| POT4     | CAGACGTGGCGATGAGAGA/CCAGGAACACTAGGCCAGT | |
| POT5     | AGTTACTTGGGTGTCAATACCCTAGTGGGACAATCG | |
## 1. Resource Table

| Unique stem cell identifier | FDCHDPe010-A-56 |
|-----------------------------|------------------|
| Alternative name(s) of stem cell line | LRP2-KO hESCs |
| Institution | Eye & ENT Hospital, Shanghai Medical School, Fudan University, Shanghai, China |
| Contact information of distributor | Jie You, 513yj45@163.com; Ling Chen, linglingchen98@hotmail.com |
| Type of cell line | ESC |
| Origin | Human |
| Additional origin info | Age: blastocyst stage<br>Sex: female, 46, XX<br>Ethnicity: N/A |
| Cell source | N/A |
| Clonality | Clonal |
| Method of reprogramming | N/A |
| Cell culture system used | mTeSR™1 |
| Genetic modification | Yes |
| Type of modification | Induced mutation |
| Associated disease | Endocytosis deficiency disease |
| Gene/locus | Gene: LRP2<br>Locus: 2q31.1 |
| Method of modification | CRISPR/Cas9 |
| Site-specific nuclease (SSN) delivery method | Plasmid transfection |
| All genetic material introduced into the cells | Cas plasmid<br>Karyotyping |
| Analysis of the nuclease-targeted allele status | Targeted PCR/sequencing |
| Method of the off-target nuclease activity surveillance | N/A |
| Name of transgene or resistance | Transient expression of Sacas9 and GFP under CMV promoter |
| Inducible/constitutive system | December 2020 |
| Data archived/stock date | N/A |
| Cell line repository/bank | N/A |
| Ethical approval | This study was approved by the ethics committee of Fudan University affiliated Eye & ENT Hospital (KJ2011-04) |