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

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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

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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.

Appendix A. Supplementary data

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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

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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 5×10^5 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, 1×10^6 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

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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.

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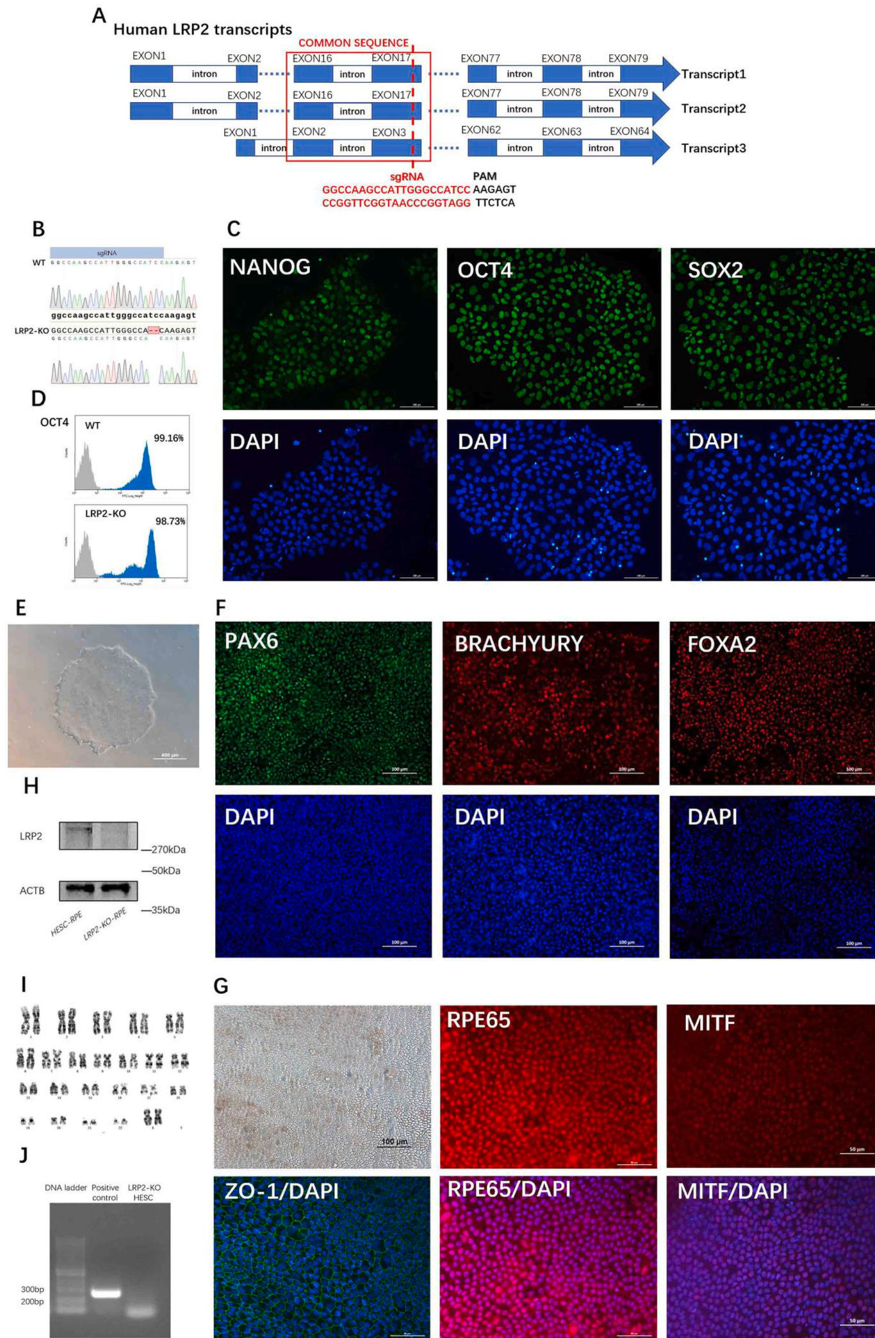

Fig. 1.

Characterization of LRP2 knockout human embryonic stem cell line (FDCHDPe010-A-56).

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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
	Quantitative analysis (i.e. Flow cytometry, RT-qPCR)	Flow cytometry: Oct4: > 98%	Fig. 1 panel D
Karyotype	Karyotype (G-banding) and higher-resolution, array-based assays (KaryoStat, SNP, etc.)	46XX, Resolution 400	Fig. 1 panel I
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site or targeted allele-specific	PCR + sequencing: Homozygous 2-bp deletion	Fig. 1 panel B, H
	PCR	WB: Completely knock- out	
	Transgene-specific PCR	N/A	N/A
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
		D5S818, D13S317, 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
	PCR-based analyses	N/A	N/A
	Southern Blot or WGS; western blotting (for knock- outs, KOs)	WB: Completely knock- out	Fig. 1 panel H
Off-target nuclease analysis-	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing	No off-target effect	Available with the author
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 (FOXA2)	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

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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# ab92494, 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# 402200, 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)	1:5000	Yeasen Cat# 33101ES60, RRID: N/A
Nuclear stain	DAPI	1:1000	Abcam Cat# ab104139, RRID: N/A
Site-specific nuclease Nuclease information	N/A	N/A	
Delivery method			
Selection/enrichment strategy	FACS	FACS	
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	GCAGTATCTGGAGAATCTCTGTTTG/ GAGTTTCCACTAAATCTTGTCATTCAGC	
gRNA oligonucleotide/crRNA sequence	LRP2	GGCCAAGCCATTGGGCCATCC	
Potential random integration-detecting PCRs	PB1	ATACGATGTTCCAGATTACGCT/ GGTGTTTCGTCCTTTCCACAAG	

Antibodies and stains used for immunocytochemistry/flow-cytometry

	Antibody	Dilution	Company Cat # and RRID
	PB2	CGTGTTTATCTCGTCAACTTGTTGG/CCAGTTTGGAACAAGAGTCCACTAT	
	PB3	CTCGAGGCGTTGACATTGAT/GTGGCACCGGTAGTTAGCC	
Top off-target mutagenesis predicted site sequencing	POT1	CTTTGCCCGGCCAAGAATTC/TCTGTCAGGCATCATGCTGGG	
	POT2	GTGGCATTCCGAATTCTGGC/GTGTTCACACCAGAGCCTGAG	
	POT3	CAGATGGTCAGAGCAGGCTC/TGATGAACCCTTGGGCCAAAG	
	POT4	CAGACGTGCCGATGAAGAGA/CCACAGGAACACTAGGCCAGT	
	POT5	AGTTTACTTGGTGTTCATTACCCA/TGGGCATTTGGAGGGACATCG	

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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 Sex: female, 46, XX 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 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
	Karyotyping
Analysis of the nuclease- targeted allele status	
Method of the off-target nuclease activity surveillance	Targeted PCR/sequencing
Name of transgene or resistance	N/A
Inducible/constitutive system	Transient expression of Sacas9 and GFP under CMV promoter
Data archived/stock date	December 2020
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)