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Knockout of the *CEP290* gene in human induced pluripotent stem cells

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Abstract

CEP290 is a principal component of the primary cilium and is important for the proper function of ciliated cells. *CEP290* mutations have been linked to numerous ciliopathies, with a wide range of phenotypic severities, but with poor genotype:phenotype correlation. Here we have used CRISPR/Cas9 technology to target the *CEP290* gene and generate a line of induced pluripotent stem cells that lack detectable CEP290 expression, but retain a normal karyotype and differentiation potential. This line of cells will be useful for the study of disorders resulting from *CEP290* mutations.

1. Resource utility

There is poor genotype: phenotype correlation with *CEP290* mutations. This line of cells lacks all detectable CEP290 expression and will be a useful comparator for functional studies of patient-derived mutations with variable pathogenicity.

2. Resource details

CEP290 is located at 12q21.32 and encodes a component of the primary cilium transition zone (Craige et al., 2010). The primary cilium is an important organelle into which a wide range of receptors and signalling molecules are segregated, and thus it is one of the primary means by which cells interact with their local environment (Gerdes et al., 2009). Mutations in *CEP290* have been linked to a spectrum of heritable disorders ranging from the blinding disease Leber's Congenital Amaurosis to multiorgan diseases such as Bardet-Biedel syndrome (Coppieters et al., 2010). The ability to derive lines of induced pluripotent stem cells from patient biopsy samples now enables investigators to more easily study the effects of *CEP290* mutations in specific cell types. Because many *CEP290* mutations are thought to be hypomorphic (Roosing et al., 2017), we sought to produce a line of *CEP290* knockout

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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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2021.102243.

stem cells that can be used as a negative control for the comparison of patient derived lines (Table 1).

A guide RNA (Fig. 1A, red text) targeting exon 9 of *CEP290* was designed using ZiFiT (http://zifit.partners.org/ZiFiT/) and inserted into pSpCas9(BB)-2A-Puro(PX459)V2.0 (Addgene). Exon 9 encodes a portion of CEP290 near the N-terminus (Fig. 1A, asterisk, adapted from Coppieters et al., 2010), at which point a truncating mutation would disrupt all known functional domains of the protein. This construct was transfected into the EP-1 line of iPSC cells, which were originally derived from the human lung fibroblast line IMR-90 (Bhise et al., 2013). After selection with puromycin and expansion, we cloned and sequenced the targeted region of both *CEP290* alleles from each candidate colony and established a line of mutant cells, designated CEIi001-A, that contained two frameshifting mutations (c.576–592del and c.584–590del, Fig. 1A). Eight potential off-target sites were sequenced from that line, and no CRISPR-Cas9 activity was detected at any of them (Supplemental Fig. S1). The cells in this line grew in compact colonies similar to the parental line, with a large nucleus:cytoplasm ratio and prominent nucleoli (Fig. 1B), and STR analysis confirmed that they are genetically identical to IMR-90 (supplemental data). The cells also had a normal human female karyotype (Fig. 1C). We demonstrated pluripotency with droplet digital RT-PCR (ddRT-PCR) assays showing expression of *SOX2* and *NANOG*, (Fig. 1D), and by immunolabeling for SOX2 and OCT4, which both had nuclear expression (Fig. 1E). The cells also tested negative for mycoplasma contamination (supplemental data).

We predicted that the frameshifting mutations in CEIi001-A would prevent expression of full-length CEP290 protein. To test this, we western blotted cell lysates from EP-1 and CEIi001-A with a C-terminal CEP290 antibody. This antibody prominently labeled a band at the predicted size in EP-1 cells, which was absent in CEIi001-A (Fig. 1F, green band). Total protein staining of the blot showed that sample loading was comparable (Fig. 1F, red).

Finally, the differentiation potential of CEIi001-A was confirmed by trilineage analysis, showing that expression of germ layer-specific genes was enriched under their respective culture conditions (Fig. 1G).

3. Materials and methods

3.1. Cell culture

EP-1 cells were a gift from Donald Zack, and were cultured in mTESR-1 media (Stem Cell Technologies) on Matrigel coated plates at 37°C in 10% CO_2, 5% O_2. Cells were routinely passaged with Accutase (Sigma) when colonies began to merge, and replated in media containing 5 μM blebbistatin (Sigma).

3.2. Gene targeting

A sgRNA sequence targeting exon 9 of *CEP290* (Fig. 1A, red text) was cloned into the BbsI site of pSpCas9(BB)-2A-Puro(PX459)V2.0 (a gift from Feng Zhang, Addgene plasmid #72988). EP-1 cells were transfected in 24-well plates with Lipofectamine STEM (ThermoFisher), and treated with 0.9 μg/ml puromycin 48 h later. After 24 h selection, cells

were grown in non-selective media for several days and then passaged at low density into a matrigel-coated 6 well plate. Colonies were picked manually and transferred to coated 24-well plates. After expansion, both alleles from the targeted region were cloned and sequenced. Potential off-target sites were identified with Cas-Offinder (http://www.rgenome.net/cas-offinder/) and sequenced.

3.3. Immunofluorescence

Cells were plated on matrigel-coated chamber slides and grown until large colonies formed. They were then fixed with 4% paraformaldehyde for 5 min and blocked in PBS containing 5% goat serum, 0.1% Tween-20 and 0.1% DMSO. Primary antibody incubation was done in blocking buffer overnight at 4°C, followed by washing and secondary antibody incubation. Cells were counterstained with DAPI and imaged on a Zeiss Imager.Z2 with Apotome. See Table 2 for antibody information.

3.4. Western blotting

Cells were scraped in RIPA buffer and sonicated. 10 μg total protein was run on a 6% polyacrylamide gel and transferred to Immobilon-FL membrane (Millipore). The membrane was first stained with REVERT total protein stain (Li-Cor), then blocked with Odyssey blocking buffer and incubated with primary antibody overnight at 4°C in blocking buffer. After washing the membrane, it was incubated with secondary antibody in blocking buffer for 1 h at room temperature. Visualization was performed with an Odyssey CLx infrared scanner (Li-Cor).

3.5. ddRT-PCR analysis

RNA was extracted with Trizol (ThermoFisher), and cDNA was synthesized with an iScript kit (Bio-Rad). ddPCR analysis was performed using SybrGreen with a QX200 droplet scanner (Bio-Rad). Primer sequences are listed in Table 2.

3.6. Karyotyping and STR analysis

Karyotyping and STR analysis were performed by Cell Line Genetics (Madison, WI).

3.7. Mycoplasma detection

Mycoplasma testing was completed by the Cell Services Core at the Cleveland Clinic Lerner Research Institute using the MycoAlert PLUS kit (Lonza).

3.8. Trilineage differentiation

Differentiation potential was assessed using the STEMdiff trilineage differentiation kit (Stemcell Technologies), in triplicate, following the manufacturer's differentiation protocol. Expression of germ layer-specific genes was measured by ddRT-PCR. Primer sequences are listed in Table 2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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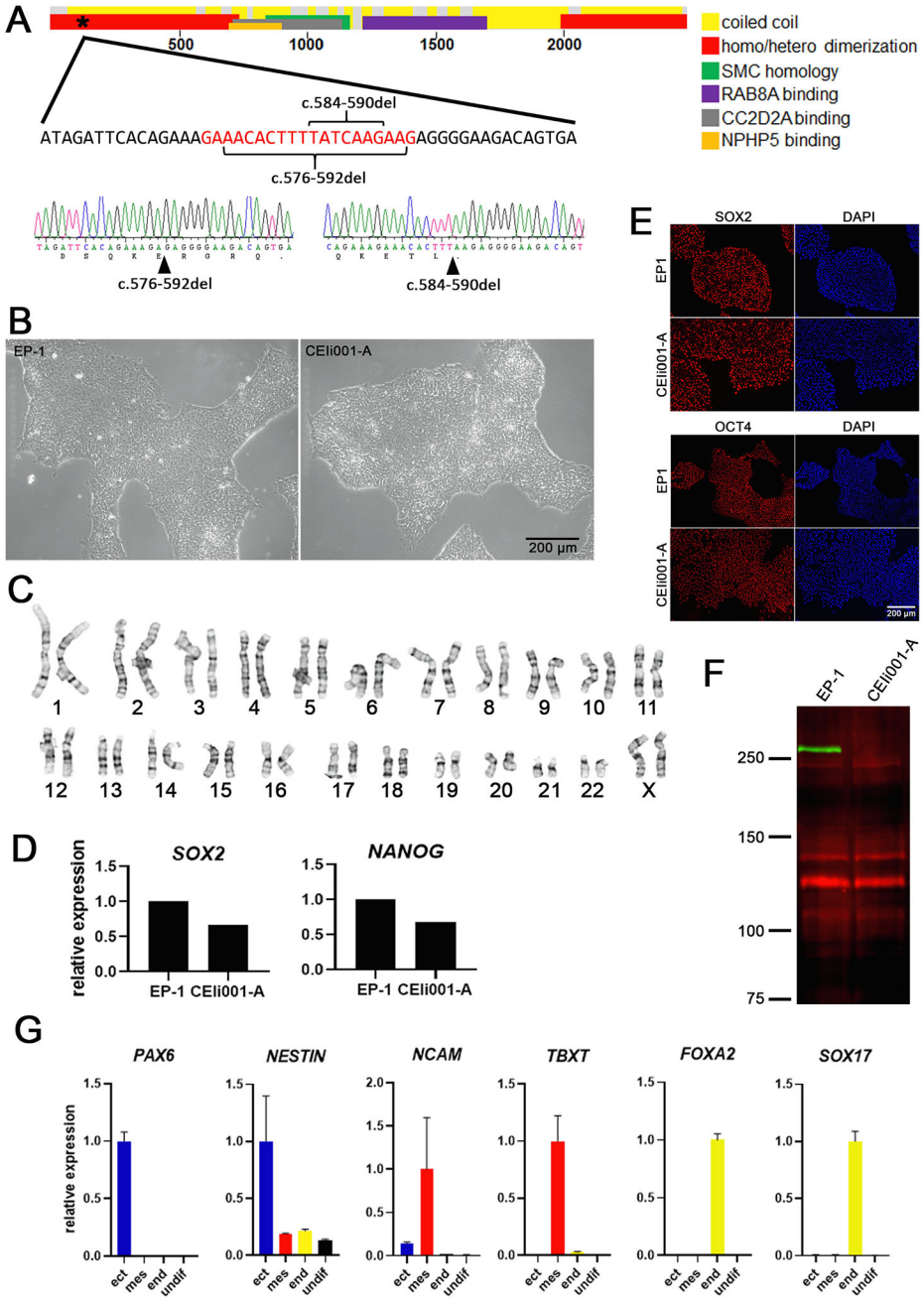

Fig. 1.

Resource Table

Unique stem cell line identifier	CEIi001-A
Alternative name(s) of stem cell line	CEP290Ex9-4
Institution	Cleveland Clinic – Cole Eye Institute
Contact information of distributor	Joseph Fogerty
Type of cell line	iPSC
Origin	human
Additional origin info	Applicable for human ESC or iPSC
	Age: Unknown
	Sex: F
	Ethnicity if known:Unknown
Cell Source	EP-1 iPSC line
Clonality	Clonal
Method of reprogramming	N/A
Genetic Modification	YES
Type of Modification	CRISPR/Cas9-induced gene knockout
Associated disease	Ciliopathies
Gene/locus	CEP290/12q21.32
Method of modification	CRISPR/Cas9
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	19 Oct. 2020
Cell line repository/bank	https://hpscreg.eu/user/cellline/edit/CEIi001-A
Ethical approval	Cell lines were used according to institutional guidelines.

Table 1

Characterization and validation

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1B
Phenotype	Quantitative analysis (ddRT-PCR)	Expression of *SOX2* and *NANOG*	Fig. 1D
	Qualitative analysis (Immunocytochemistry)	Positive staining for pluripotency markers SOX2, OCT4	Fig. 1E
Genotype	Karyotype (G-banding) and resolution	46XX, Resolution 500	Fig. 1C
Identity	Microsatellite PCR	N/A	N/A
	STR analysis	16/16 loci matched	Available from the authors.
Mutation analysis (IF APPLICABLE)	Sequencing	Compound heterozygous Allele 1: c.576-592del Allele 2: c.584-590del	Fig. 1A
	Western Blot	Cep290 protein is not detectable in mutant cells	Fig. 1F
Microbiology and virology	Mycoplasma	Negative	Supplementary data
Differentiation potential	Directed differentiation	Expression of germ layer-specific genes was enriched under their respective culture conditions.	Fig. 1G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 2

Reagents details

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Marker	OCT4	1:100	DSHB #PCRP-POU5F1-1D9-S RRID: AB_2618969
Pluripotency Marker	SOX2	1:100	Cell Signaling Technology #3579T RRID: AB_2195767
Knockout confirmation	CEP290	1:1000	Abcam #ab84870 RRID: AB_1859782
Secondary antibody (ICC)	Goat anti-mouse Alexa-568	1:500	ThermoFisher #A11004 RRID: AB_2534072
Secondary antibody (ICC)	Goat anti-rabbit Alexa-568	1:500	ThermoFisher #A11036 RRID: AB_10563566
Secondary antibody (WB)	IRDye 800CW Goat anti-rabbit	1:10000	Li-Cor #925-32211 RRID: AB_2651127
Primers			
	Target	**Forward/Reverse primer (5′-3′)**	
Pluripotency Marker (ddPCR)	*SOX2*	AGAAGAGGAGAGAGAAAGAAAGGGAGA/ GAGAGAGGCAAACTGGAATCAGGATCAAA	
Pluripotency Marker (ddPCR)	*NANOG*	GAACTCTCCAACATCCTGAACCT/TCTGCGTCACACCATTGCTAT	
Ectoderm Marker (ddPCR)	*PAX6*	GTCCATCTTTGCTTGGGAAA/TAGCCAGGTTGCGAAGAACT	
Ectoderm Marker (ddPCR)	*NESTIN*	CAGGGGCAGACATCATTGGT/CACTCCCCCATTCACATGCT	
Mesoderm Marker (ddPCR)	*NCAM*	ATGGAAACTCTATTAAAGTGAACCTG/ TAGACCTCATACTCAGCATTCCAGT	
Mesoderm Marker (ddPCR)	*TBXT*	GCTGTGACAGGTACCCAACC/CATGCAGGTGAGTTGTCAGAA	
Endoderm Marker (ddPCR)	*FOXA2*	GGAGCGGTGAAGATGGAA/TACGTGTTCATGCCGTTCAT	
Endoderm Marker (ddPCR)	*SOX17*	GTGGACCGCACGGAATTTG/GGAGATTCACACCGGAGTCA	
Housekeeping Gene (ddPCR)	*GAPDH*	TCCAAAATCAAGTGGGGCGAT/TTCTAGACGGCAGGTCAGGTC	
Targeted mutation analysis/ sequencing	*CEP290*	ACTTTGTCAGGATATTATTGACTACCA/TTTAGACAACTGTGATCGGTAGT	
Potential off-target sequencing	*POT1*	TCCTCGAGAATTGTGCACCT/AACATCCAACCACACTGCGA	
Potential off-target sequencing	*POT2*	GTCAGTCCTGGGCAGAGAAC/CTGTCTCTTGCTGCTTTGCG	
Potential off-target sequencing	*POT3*	GGCAGTGCCTTGGAGAGAAT/CCCGTTGCTCATTTCCTCCT	
Potential off-target sequencing	*POT4*	AGCAGTCTGTTACAGCAGCA/TTCCCTTTTTCTGAGCCCCC	
Potential off-target sequencing	*POT5*	TAGGAGCTTCGACTTGCCAC/TAGGAGCTTCGACTTGCCAC	
Potential off-target sequencing	*POT6*	GGTACCTGAATGGCCAGTCA/TGCCACAACAAAGACATATCCC	
Potential off-target sequencing	*POT7*	GGCCCCTTTTGCCTACTTCT/CCATTGGGCTAGGGAATGGT	
Potential off-target sequencing	*POT8*	TGGATGCAGAAATGGAGGCT/ATGTACCTGCTGGTTGGCAT	