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 (Craigie 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...
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 CEi001-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 CEi001-A would prevent expression of full-length CEP290 protein. To test this, we western blotted cell lysates from EP-1 and CEi001-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 CEi001-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 CEi001-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% CO₂, 5% O₂. 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.
Acknowledgements

The authors thank Donald Zack and Alyssa Kallman for their expertise and technical assistance. This work was supported by NIH grants EY017037 and EY030574 (to BDP), a Doris and Jules Stein Professorship Award from Research to Prevent Blindness (BDP), and a Knights Templar Eye Foundation Career Initiation Grant (JF). Additional support to the Cole Eye Institute was provided by the National Eye Institute (P30-EY025585), Research to Prevent Blindness, and the Cleveland Eye Bank Foundation.

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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

**Antibodies used for immunocytochemistry/flow-cytometry**

| Antibody                    | Dilution | Company Cat # and RRID                  |
|-----------------------------|----------|----------------------------------------|
| Pluripotency Marker         | OCT4     | DSHB #PCRP-POU5F1-1D9-S RRID: AB_2618969 |
| Pluripotency Marker         | SOX2     | Cell Signaling Technology #3579T RRID: AB_2195767 |
| Knockout confirmation       | CEP290   | Abcam #ab84870 RRID: AB_1859782         |
| Secondary antibody (ICC)    | Goat anti-mouse Alex-568 | ThermoFisher #A11004 RRID: AB_2534072 |
| Secondary antibody (ICC)    | Goat anti-rabbit Alex-568 | ThermoFisher #A11036 RRID: AB_10563566 |
| Secondary antibody (WB)     | IRDye 800CW Goat anti-rabbit | Li-Cor #925-32211 RRID: AB_2651127 |

**Primers**

| Target                     | Forward/Reverse primer (5'‑3') |
|----------------------------|--------------------------------|
| Pluripotency Marker (ddPCR) | SOX2 AGAAGAGGAGAGAAAAGAGG/GAGAGGCAAAACTGGAATCAGGATCAA |
| Pluripotency Marker (ddPCR) | NANOG GAACCTCTCAACATCTGACACCT/TTGCGTCACACCATTGCTAT |
| Ectoderm Marker (ddPCR)     | PAX6 GTCCATCTTTGCTTGGGAAA/TAAGGACGTTCACACCAGAGT |
| Ectoderm Marker (ddPCR)     | NESTN CAGGGGCAGACATCATTTGCGTC/CACTCTCCCCCTACACATGCT |
| Mesoderm Marker (ddPCR)     | NCAM ATGGAAACTCTATTAAGTGAACACGG/ TAGACCTCATACGACATTAGT |
| Mesoderm Marker (ddPCR)     | TBX7 GCTGTGACAGGTACCAAGCC/ CATGCAAGGTGATGTCAGAAA |
| Endoderm Marker (ddPCR)     | FOXA2 GGAGCGGGTGAAGATGGA/TAAGGACGTTCACACCAGAGT |
| Endoderm Marker (ddPCR)     | SOX17 GTGGACCGCAGGAAATTG/GGAGATTCACACCGGAGT |
| Housekeeping Gene (ddPCR)   | GAPDH TCCAAAATCAAGTGCCAGCG/TTATTAGACGGCGAGT |
| Targeted mutation analysis/ sequencing | CEP290 ACTTTGTCAGATATTATGACTACA/TTAGACAACGTCAGGATAGT |
| Potential off-target sequencing | POT1 TCCTCGAAGAATTGTCACCT/AAACATCCCAACACACTGCGA |
| Potential off-target sequencing | POT2 GTCACTCTGCGAGGAGAAAC/CTGTCCTTTGTCGGT |
| Potential off-target sequencing | POT3 GTCGGGCAGAGAAATGCA/ACGCCCTTCCCTTCCTTCCTTC |
| Potential off-target sequencing | POT4 ACCACGTGTTACAGCGAC/TTCTGCTTTTCGAGCCC |
| Potential off-target sequencing | POT5 TAGGAGCTTCAGCTCGAC/AGAGACGTCACACCGGAGT |
| Potential off-target sequencing | POT6 GGTACCTGAAATGCCAGTCA/AGCCACACAAAGACATATCCC |
| Potential off-target sequencing | POT7 GCCTGCTTTTGCTACTTCT/CACATTGGGCTAGAGGATG |
| Potential off-target sequencing | POT8 TGGATGCAAAATGGGAGCT/ATGTACCTGCTGAGGAGT |

*Stem Cell Res. Author manuscript; available in PMC 2021 May 17.*