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Supplemental Information

Targeted Gene Correction of Laminopathy-Associated *LMNA* Mutations in Patient-Specific iPSCs

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Supporting Information

Contains 1 Supplemental Figure and Figure Legend, 1 Supplemental Table, 1 Supplemental Movie, Supplemental Experimental Procedures and Supplementary References

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Figure S1 shows the generation and the characterization of cHGPS-iPSCs, AWS-iPSCs, cAWS-iPSCs and gene-targeted OE-MSCs. This figure supports data presented in main Figures 1, 2 and 3.

Table S1 shows Replicate Error analysis of SNP genotyping data. This table supports data presented in main Figure 2. Available online.

Movie S1 shows contractile cardiac tissue with pacemaker activity *in vitro* differentiated from cHGPS-iPSCs. This movie supports data presented in main Figure 2. Available online.

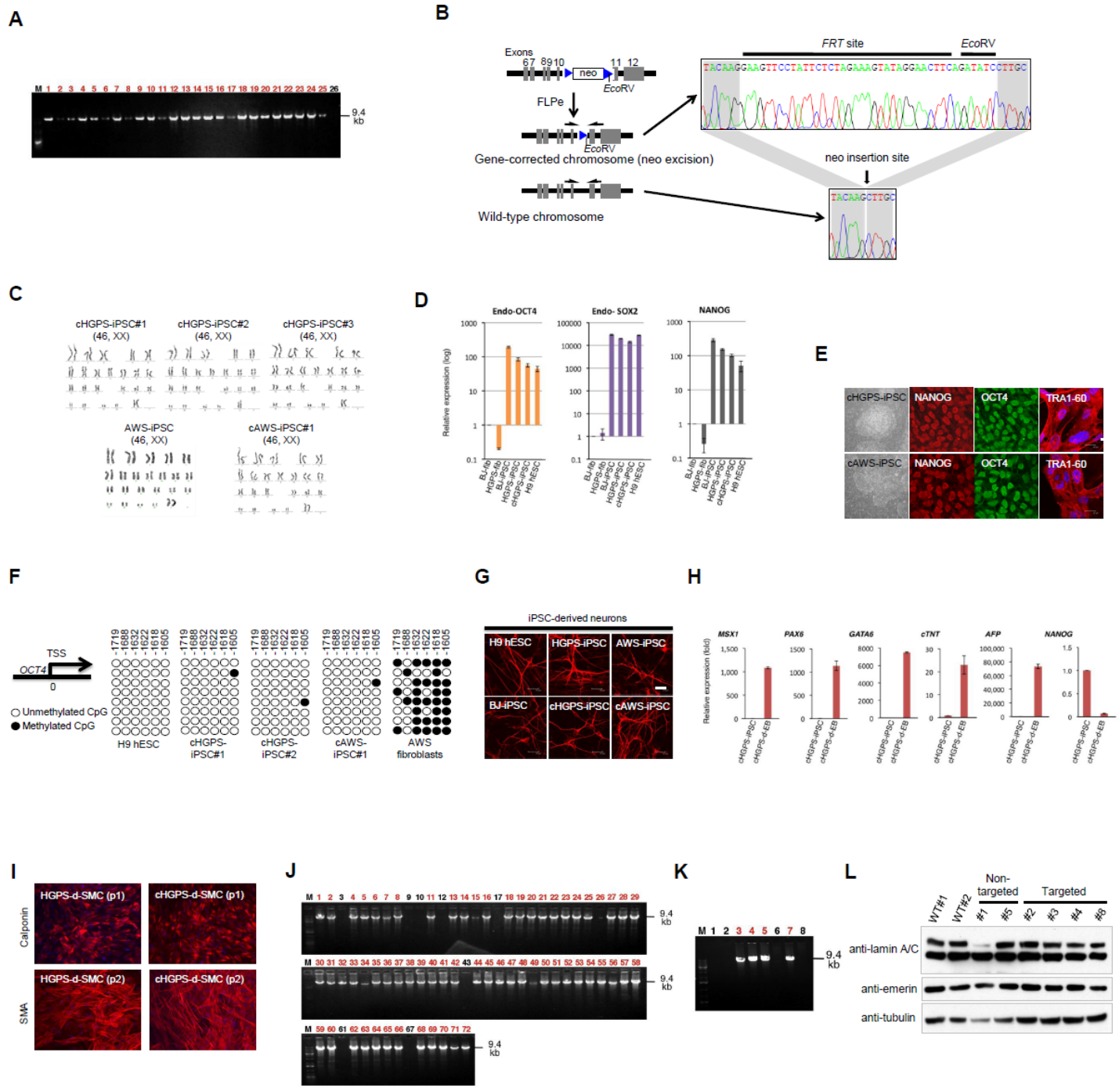

Figure. S1. Characterization of the indicated iPSC and MSC lines. (**A**) PCR analyses of HGPS-iPSCs targeted with the *LMNA*-c-HDAdV (MOI=3, single cell) using 3' primer pair (P3 and P4; 9.4 kb). M, DNA ladder. Gene-targeted clones are indicated in red. (**B**) Sequencing results showing that neomycin-resistant cassette (neo) was successfully removed from intron 10 of *LMNA* gene in the cHGPS-iPSCs. Schematic demonstration of a 41 bp sequence left in intron 10 of the *LMNA* locus after neo removal. (**C**) Karyotypic analysis of the indicated iPSC lines showing normal karyotypes. (**D**) Quantitative RT-PCR analysis of endogenous (endo) pluripotent genes in the BJ-iPSCs, HGPS-iPSCs, and cHGPS-iPSCs. H9 hESCs were used as a positive control. Data are shown as mean±s.d. n=3. (**E**) Immunofluorescence analyses performed in

cHGPS-iPSCs and cAWS-iPSCs showing expression of pluripotent markers Nanog, OCT4, and TRA1-60. Bar, 20 μm. (**F**) DNA methylation status of the *OCT4* promoter in the indicated iPSC lines. (**G**) *In vitro* differentiation of the indicated pluripotent stem cells into neurons. Neurons were immunostained with anti-MAP2 antibody. Bar, 20 μm. (**H**) Quantitative RT-PCR analysis of the expression of genes from three germ layers including *MSX1*, *PAX6*, *AFP*, *GATA6*, and *cTnT*, and pluripotent gene *NANOG* in undifferentiated cHGPS-iPSCs and differentiated cHGPS-iPSCs (EB, day 18). Data are shown as mean±s.d. n=3. (**I**) Immunostaining of calponin and SMA in iPSC-derived SMCs. Nuclei were visualized with Hoechst33342 stain (blue). (**J**) PCR analyses of AWS-iPSCs targeted with the *LMNA*-c-HDAdV (MOI=3, single cell) using 3' primer pair (P3 and P4; 9.4 kb). M, DNA ladder. Gene-targeted clones are indicated in red. (**K**) PCR analyses of OE-MSCs targeted with the *LMNA*-c-HDAdV (MOI=10, adherent cell) using 3' primer pair (P3 and P4; 9.4 kb). M, DNA ladder. Gene-targeted clones are indicated in red. (**L**) Immunoblotting analysis of lamin A/C and emerin in gene-targeted and non-targeted OE-MSC lines with the *LMNA*-c-HDAdV (MOI=10, adherent). Tubulin was used as a loading control. All iPSCs employed represent high-passage iPSCs (>30). This figure supports data presented in main Figures 1, 2 and 3.

Supplemental Experimental Procedures

Cell culture and differentiation. Human Atypical Werner Syndrome (AWS) fibroblast line AG04110 was purchased from Coriell Cell Repository. This fibroblast line bears heterozygous mutation (A1733T) in exon 11 of the *LMNA* gene, causing a missense mutation (E578V). The fibroblasts were cultured in DMEM (Invitrogen) containing 15% fetal bovine serum (FBS, Gemini), 0.1mM non-essential amino acids (Invitrogen), 1mM glutamax (Invitrogen), and 1mM sodium pyruvate (Invitrogen). OE-MSC line was described and characterized elsewhere (Delorme et al., 2010). H9 hESCs (WiCell Research) and iPSCs were cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs) and/or matrigel as previously described (Liu et al., 2011). Stem cell differentiation into different lineages was performed as previously described (Liu et al., 2011). Gene targeting experiments were started with cells at passage 30. Passage number after correction could not be precisely monitored due to the inherent selection processes required for gene targeting.

iPSCs Generation. Production of retroviruses and iPSCs generation was performed as previously described (Liu et al., 2011).

Antibodies and retroviral vectors. All antibodies used were described previously (Liu et al., 2011). The retroviral expression vectors pMSCV-Flag-hOct4, pMSCV-Flag-hSox2, pMSCV-Flag-hKlf4, and pMSCV-Flag-cMyc T58A were described previously (Aasen et al., 2008).

mRNA and protein analysis. Total RNA was isolated with Trizol reagent (Invitrogen), and cDNA were synthesized with High capability RNA-to-cDNA Mater Mix (Invitrogen). Quantitative RT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences are described previously (Liu et al., 2011). Immunoblotting analysis was performed based on the previously described method (Liu et al., 2011).

Isolation of the gene-targeted and gene-corrected iPS clones. To infect *LMNA*-c-HDAdV, 3 x10^6 feeder-free cultured HGPS-iPSCs or AWS-iPSCs were dissociated by TrypLE (for single cell; Invitrogen) or dispase (for clump; Invitrogen), and resuspended in 1ml of MEF-conditioned medium containing 10 μM ROCK inhibitor Y-27632 (Sigma-Aldrich). The cells were infected with *LMNA*-c-HDAdV at MOIs of 3, 30 or 300 btu/cell at 37°C for 1 h, and after brief centrifugation resuspended in 10 ml of MEF-conditioned medium containing 10 μM Y-27632. The cells were then plated onto 100 mm dishes precoated with neomycin-resistant MEFs. Two days after infection, G418 (50 μg/ml; Invitrogen) was added to the medium to start the positive selection. After 2-3 weeks, G418-resistant colonies were transferred to 96-well plates, where the negative selection with GANC (2 μM; Invitrogen) was carried out for another 5-7 days. G418/GANC double-resistant clones were collected for further characterization. Gene-targeting efficiency was determined by long PCR-screening of genomic DNA from the drug-resistant clones with the following primers (P1,5'-GCAGACCCACAGACTAGCCAAGAAACCAAGCAGT-3';P2,5'-CCCCAAAGGCCTACCCGCTTC CATTGCTCA-3';P3,5'-CTACCTGCCCATTCGACCACCAAGCGAAACATC-3';P4,5'-GGTCCTCTGCCTC

ACTTTTCCCTCATCCCAT-3’; also see Fig. 1A) with LA Taq Hot Start Version (TAKARA). The long PCR cycling was a 1-min initial denaturation at 94 °C, 14 cycles with 20 s denaturation at 94 °C and a 10-min annealing and an extension at 68 °C, 21 cycles with 20 s denaturation at 94 °C and a 10-min +5 sec/cycle annealing and an extension at 68 °C and a final extension at 68 °C for 10 min. The structure of the *LMNA* locus was further analyzed by Southern blotting with the genomic DNAs extracted from the PCR-positive clones. To determine the gene-correction efficiency and SNPs-correction events, genomic DNAs were extracted from the gene-targeted clones. Exon 11 or its downstream region of *LMNA* were PCR-amplified with the following primers:

a) mutation site, 5’-GATATCCTTGCTCCCGTTCTCTCTTCTTTTCCTCTTAAGCT-3’ and 5’-CTCGAGAGGTAGCATCTCCCCCATCCCTCACACTC-3’

b) SNP site1, 5’-GCAGATATGGGGTCTTAGATGTCAGCGAGGGA-3’ and 5’-TGAAATGTGGCTGCTGAGTGCTGACTTGTGCTGT-3’

c) SNP site 2, 5’-CATGTTAGCCAGGATGGTCTCG-3’ and 5’-TGAGGCACAAGAATCACTGGA-3’

with Ex Taq Hot Start Version (TAKARA), following the manufacturer's protocol. Amplicons were sequenced by ABI 3730 sequencer (Applied Biosystems).

Excision of the neomycin-resistant cassette in gene-corrected iPS clones. Gene-corrected iPSCs cultured on Matrigel (BD Biosciences) were transfected with pCAG-Flpe vector (addgene 13787) using FuGENE HD (Roche). Three days later, the cells were individualized and plated onto MEF feeder cells at a density of 300-3000 cells/75cm^2. After 2 weeks, the emerging colonies were picked and expanded. The removal of the neomycin-resistant cassette was verified by various approaches including G418 sensitivity test, PCR confirmation, and DNA sequencing.

***LMNA* gene-targeting in OE-MSCs.** For suspension infection, individualized OE-MSCs were infected with *LMNA*-c-HDAdV at MOIs of 0.1-10 btu/cell in 300 μl of transduction solution (PBS with 680 μM $MgCl_2$ and 500 μM $CaCl_2$) at 37°C for 1 h. After centrifugation at 1500 rpm for 3 min, cells were resuspended in 2 ml of MSC medium, DMEM/F12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gemini) and 1mM glutamax (Invitrogen), and plated in 1 well of 6-well plates. For adherent infection, OE-MSCs were plated in 1 well of 6-well plates 24 h before infection, and the cells were infected with *LMNA*-c-HDAdV at MOIs of 10 and 30 btu/cell in 300 μl of transduction solution at 37°C for 1 h, and then continued to be cultured in MSC medium. G418 selection (200-450 μg/ml) was started 48 h after infection. Upon confluency, cells were subcultured and maintained in G418-containing medium for another 3-4 weeks. Survival colonies were picked and further expanded. Gene-targeting efficiency at *LMNA* locus was determined by PCR.

Southern blotting. Ten μg of genomic DNA for each sample was digested with *Eco*RV (NEB) and subjected to

4 h at 50 V on a 0.65% agarose gel electrophoresis. The gel was subsequently incubated in depurination buffer (0.25 M HCl) for 10 min followed by 30-min incubation in denaturation buffer (1.5 M NaCl, 0.5M NaOH). The DNA was then blotted overnight onto a Hybond XL (GE Healthcare) by capillary transfer in denaturation buffer. The membrane was then incubated in neutralization buffer (0.5 M Tris-HCl [pH 7.4], 1.5 M NaCl) for 20 min followed by UV crosslinking. The 5' and 3' probe was amplified from genomic DNA with the following primers (5' probe, 5'-CCACATTTCTCTATTTCCCCGTGA-3' and 5'-AGCCCCTTAACTCTCTATCTGGT-3'; 3' probe, 5'-GGAACCTTCTCTGCCCCAATGCC-3' and 5'-GCCATGCCCAGTCACCTACAACTCC-3') with Ex Taq Hot Start Version (TAKARA), following the manufacturer's protocol. The probes were labeled with the dCTP [α-^{32}P] (MP Biomedicals), and Southern hybridization was performed following the standard protocol.

DNA microarray and Bioinformatics analysis. Total RNA were extracted from two independently prepared cultures of each cell line using Trizol Reagent (Invitrogen) and purified by RNeasy Mini Kit (QIAGEN). The Affymetrix GeneChip microarray was performed by the Functional Genomics Core Facility at The Salk Institute for Biological Studies according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). Briefly, 100 ng of total RNA of each sample were processed using Affymetrix GeneChip 3' IVT Expression Kit following the manufacturer's protocol. For each sample, 12.5 μg fragmented and labeled aRNA were hybridized to the Affymetrix HG-U133A 2.0 chips. Expression signals were scanned on an Affymetrix GeneChip Scanner 3000 7G. The analysis of the data was performed using affy package in R-Bioconductor. Briefly, .CEL files were imported in R-bioconductor for preprocessing and normalization. dChip software were used to perform hierarchical clustering on RMA-normalized probeset intensity values. In addition, a principal component analysis was performed on RMA-normalized probeset intensity values using the Genesis software. Microarray data have been deposited in NCBI-GEO with the accession number GSE28607.

Bisulfite sequencing of *OCT4* promoter. Bisulfite conversion of DNA was carried out using the Zymo EZ DNA Methylation-direct Kit (Zymo Research) following the manufacturer's recommendations. About two thousand cells were used as starting material per sample. A genomic fragment of the *OCT4* promoter was amplified with previously published primers using the 2x Zymo Taq Premix per manufacturer instructions (Liu et al., 2011). PCR products were purified by gel extraction using QIAquick columns, and subsequently cloned into the pCR2.1-TOPO vector (Invitrogen). Ten clones from each sample were sequenced with the M13 universal primer.

Genome-wide DNA methylation analysis. The detailed protocol has been described previously (Deng et al., 2009). Briefly, genomic DNA from the cell lines (AWS-iPSC, cAWS-iPSC, HGPS-iPSC, cHGPS-iPSC#1, cHGPS-iPSC#40) was bisulfite converted with the Zymo EZ-DNA Methylation Gold Kit (Zymo Research) following the manufacturer's instructions. The conversion rates were >99%. Approximately 60,000 genomic regions (~19Mb in total size), included known differentially methylated regions (DMRs), DNase hypersensitive sites, insulators, and imprinted genes were captured from bisulfite converted DNA with a library of ~300,000

bisulfite padlock probes, followed by Illumina sequencing (Deng et al., 2009). For hierarchical clustering, four published methylation sequencing data sets (H9-hESC, BJ-fibroblast, BJ-iPSC, HGPS-fibroblast) that we previously generated using the same method (Liu et al., 2011) were included as controls.

SNP Genotyping. SNP Genotyping was performed on the Illumina OmniQuad v1 BeadArray, which covers 1,140,419 SNPs across the human genome. 1 μg input genomic DNA, quantified using the PicoGreen reagent (Invitrogen, Inc.), was amplified and labeled according to the manufacturer's instructions. The labeled product was hybridized to the array and scanned on a BeadArray Reader (Illumina, Inc.). Genotyping calls were made using GenomeStudio (Illumina, Inc.), using the standard cluster files provided by the manufacturer. The GenCall threshold was set to 0.15, and the call rates were between 0.9947 and 0.9975.

Replicate Error analysis. We used the Replicate Error Analysis function of GenomeStudio (Illumina, Inc.) to evaluate the relatedness among samples. Samples from the same individual will have a replicate error rate of <0.01%, those from full siblings will have a replicate error rate of 10-12%, and unrelated individuals will have a replicate error rate of 18-23% (from dataset published in (Laurent et al., 2011)). The replicate error rates between HGPS-fibroblasts and cHGPS-iPSCs was 0.0077%, indicating that these cell lines were from the same individual. The replicate error rate between HGPS-fibroblasts and H9 hESCs was 19.6186%, and between HGPS-fibroblasts and control BJ-iPSCs was 20.3745%, and between H9 hESCs and control BJ-iPSCs was 20.1842%, indicating that these three cell lines were unrelated. The reference genome used was NCBI 37.

Copy Number Variation Assessment. In order to identify new duplications and deletions that arose between HGPS-fibroblast and cHGPS-iPSC samples, we performed CNV calling and replicate error analysis on the SNP Genotyping data. Data preprocessing was performed in BeadScan (Illumina, Inc.). Data cleaning, SNP calling, and replicate error identification was performed in GenomeStudio (Illumina, Inc.). The CNVPartition v2.4.4 plugin for GenomeStudio was used as the CNV-calling algorithm to detect duplications, with a confidence threshold of 100, a minimum homozygous region size of 1 million bp, minimum probe count per call of 10, and logR ratio smoothing set to "true". For the cHGPS-iPSC sample, the only duplication detected was at chr7:141322-162448, and this same duplication was detected in the parental HGPS-fibroblast line (Fig. 2B). We did not use CNVPartition to detect deletions, as we have previously found that deletions called by CNVPartition could be validated by qPCR less than 40% of the time (Laurent et al., 2011). Since we were interested in identifying new deletions in cHGPS-iPSCs compared to HGPS-fibroblasts, we were able to use the replicate error report calls to identify deletions, using a cutoff of $\geq$3 contiguous heterozygous-to-homozygous replicate errors. In fact, we did not identify even 2 such contiguous replicate errors (Table S1).

Supplementary References

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