Supporting information (SI)

CRISPR/Cas9-based targeted genome editing for correction of recessive dystrophic epidermolysis bullosa using iPS cells

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SI Materials and Methods

Ethics Statement. Informed consent was obtained from all subjects, and approval for this study was provided by the Institutional Review Board of Columbia University in accordance with the Declaration of Helsinki.

Isolation and culture of fibroblasts. Primary human fibroblasts were obtained from skin biopsies of two RDEB patients and healthy control patients. One patient suffers from RDEB caused by a homozygous *COL7A1* c.2470insG mutation in exon 19 (1, 2), which causes a frameshift and a premature termination codon on both alleles leading to attenuated basement membrane zone expression of C7. The other patient suffers from RDEB caused by heterozygous *COL7A1* c.2470insG mutation in exon 19 and c.3948insT mutation in exon 32 (1). The c.3948 mutation results in a premature termination codon and an almost complete absence of basement membrane zone expression of C7. Fibroblasts isolated from these patients were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Invitrogen). The cells were grown at 37°C and kept at 5% CO_2.

Generation and characterization of feeder-free and integration-free iPSCs. In this study, a new method to generate feeder-free and integration-free induced pluripotent stem cells (iPSCs) from fibroblasts was established with great potential for therapeutic applications. Integration-free iPSCs from fibroblasts were generated using transfection of an integration-free episomal plasmid, as described previously (3). To generate iPSCs from fibroblasts without feeder cells, $2x10^6$ fibroblasts were seeded into 10 cm^2 culture dishes and used in 4 days for electroporation. Fibroblasts were cultured in complete DMEM medium with 10% FBS and 1% penicillin-streptomycin for 4 days. On day 0 and day 3, cells were harvested and electroporated with reprogramming vectors

(pCXLE-hOCT3/4-shp53-F: OCT3/4 and p53 shRNA, pCXLE-hSK: SOX2 and KLF4, pCXLE-hUL: L-Myc and LIN28, pCXWB-EBNA1: EBNA1; Addgene plasmids # 27077, 27078, 27080, and 37624) encoding the transcription factors. The transfection into fibroblasts was performed by electroporation with the Amaxa Nucleofector 2 Device (Lonza) using the Nucleofector Kit for normal human fibroblasts. Transfected fibroblasts were cultured with TeSR™-E7™ (StemCell Technologies), a serum-free, low-protein, and xeno-free medium that was developed for reprogramming fibroblasts with episomally-delivered reprogramming vectors into iPSCs. The iPSCs were maintained in fully defined Essential 8 (E8) medium (Gibco) introduced into the cells at day 13, where they show morphological changes indicative of reprogramming. Around 21 days post-transfection, iPSC colonies with a characteristic morphology were mechanically picked up and plated on vitronectin (Gibco) coated plates for expansion and characterization. The iPSCs were cultured with E8 media supplemented with RevitaCell Supplement (Gibco) for the first 24 hours after passaging. Gentle cell dissociation reagent (StemCell Technology), an enzyme-free reagent, was used for the dissociation of iPSCs into cell aggregates for routine passaging and expansion. To confirm their pluripotency, the iPSCs were analyzed for expression of stem cells markers, teratoma formation, karyotype analysis, and differentiation capacity *in vitro* as described previously (3, 4).

Trilineage differentiation of iPSCs. To confirm the *in vitro* differentiation capacity of iPSCs into all three germ layers: ectoderm, mesoderm, and endoderm, commercially available STEMdiff™ Trilineage Differentiation Kit was used (StemCell Technologies) following the manufacturer's protocol. This kit includes specialized, complete media and monolayer-based protocols to perform parallel *in vitro* directed differentiation

experiments for each germ layer. After differentiation, the cells were analyzed through ICC for the appropriate germ layer markers as suggested in the kit.

RNA, DNA Isolation, PCR and RT-PCR condition. Total RNA was isolated from feeder-free generated iPSCs using the Qiagen RNeasy Mini Kit and treated with DNase (Invitrogen) to remove gDNA contaminations. cDNA was synthesized from RNA using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Genomic DNA was extracted using Quick Extraction DNA-Solution (Epicenter) for PCR analysis. Approximately 10 ng of the genomic DNA templates were used in all PCRs. Following amplification, the following program was used for PCR and RT-PCR reactions: cycle 1: 95 °C for 2 min; cycles 2-12: 95 °C for 20 s, 75-55 °C for 30 s, 72 °C for 1 min; cycles 13-31: 95 °C for 20 s, 55 °C for 30 s, 72 °C for 1 min; cycle 32: 72 °C for 10 min. All primers used in this study are previously described [12].

CRISPR/Cas9 gene corrections using plasmid-based method gRNA design and assembly into the plasmid. The 19 bp long gRNA, designed using Deskgen^{TM} online software, used for targeting the *COL7A1* c.2470insG mutation in exon 19 (5'-*GGACCGGTCCGCCGGGGTAC*-3') was incorporated into two complementary 100-mer oligonucleotides and cloned into a plasmid (Addgene # 41824) as previously described(5, 6). Briefly, each 100-mer oligonucleotide was reconstituted to 100 mM in water, mixed with an equal volume, and annealed in a thermocycler (95 °C, 5 min; Ramp to 4 °C, 0.1 °C/s). To prepare the destination vector, we linearized the gRNA cloning vector using AfIII restriction enzyme (New England Biolabs) and purified the linear DNA fragment. Next, 10 ng of the purified 100 bp DNA fragment was annealed with 100 ng destination backbone via Gibson assembly reaction mix (New England Biolabs) at 50 °C for 30 min (7). The gRNA assembly reaction mix was directly

processed for bacterial transformation to colonize individual assemblies. The human codon optimized pCAGGS-Cas9-mCherry was used for gene-editing experiments (a gift from the Stem Cell Core Facility at Columbia University). To generate the human codon optimized pCAGGS-Cas9-mCherry, the Cas9 from Addgene (# 41815) was cloned into the pCAGGS-mCherry plasmid from Addgene (#41583) to render a pCAGGS-Cas9-mCherry by Martin Jacko from Hynek Wichterle's laboratory at Columbia University.

Design of ssDNA repair template. The 178-nt ssDNA repair templates (PAGE-purified, IDT) were designed with homologous genomic flanking sequence centered around the targeted mutation c.2470insG in exon 19 of *COL7A1*(*5'GGTCCCCGACAAGTGCAGTCACTCGCACTGAGTAGCTGACTCCACC TTCGAGACCCCGGATCTCTGCAGAGTCTGTGTTTCCTGGGAGTATCTGGTGTCT CATGGGGCCGCCTGGCCAGGTGGGCATACAGCAATGGTTAGGGGTGAGCAGT CCCAGCCAGGAAGGACAGGGGTGGCG-3'*) and contained corrected *COL7A1* sequences in exon 19 and CRISPR/Cas-blocking mutations. The CRISPR/Cas-blocking silent decoy (does not alter the amino acid sequence) mutation was selected based on codon-usage of the edited gene by changing the codon to another codon already used in the same mRNA for the respective amino acid.

Gene-editing of *COL7A1* iPSCs by ssDNA with plasmid-based method. For gene editing, $2x10^6$ of single, patient-derived iPSCs harboring the *COL7A1* c.2470insG mutation in exon 19 iPSCs were electroporated with 2.5 ug pCAGGS-Cas9-mCherry, 7.5 ug of plasmid containing *COL7A1* specific gRNA, and 15 ug of ssDNA repair donor template using the Amaxa Nucleofector 2 Device (Lonza) transfection system with Nucleofector Kit for human stem cells. The iPSCs were then recovered in E8 medium supplemented with RevitaCell Supplement for 24 hr after electroporation. 24 hr post-

transfection, the mCherry-positive iPSCs were collected via FACS, plated at a low density in 6-well plates, and cultured for 10 days until iPSC colonies were ready to be picked.

CRISPR/Cas9 gene-corrections using Cas9-sgRNA Ribonucleprotein (RNP) complex-based method. gRNAs were designed using the ZiFiT Targeter 4.2 (http:/zifit.Partners.org) online software. The selected gRNAs were tested for activity and those with the best activity were used for gene editing studies. The 19 bp gRNAs sequences targeting the *COL7A1* c.2470insG mutation in exon 19 was *(GGGAAACACACTCTGCAG*) and the c.3948insT mutation in exon 32 was (*TGGCCGCGCCGGGAATC*). To target the *COL7A1* gene, we used site-specific chemical modifications synthetic single guide RNAs (sgRNAs) (Synthego, Inc) and GMP grade *SpyFi* Cas9 Nucleases™ (Aldevron). The site-specific chemical modifications sgRNA provides greater stability and decreased activation of the cellular innate immune response, resulting in superior gene editing. The reagents were prepared following the instructions obtained from the manufacturer. The 133-nt ssDNA repair templates (PAGE- purified, IDT) were designed with asymmetric homologous genomic flanking sequence around the targeted mutations(8). To correct c.2470insG mutation in exon 19 of *COL7A1,* ssDNA containing corrected *COL7A1* sequences in exon 19 was used (5'*CCACCCCTGTCCTTCCTGGCTGGGACTGCTCACCCCTAACCATTGCTGTATG CCCACCTGGCCAGGCGGCCCCATGAGGCACCAGATACTCCCAGGAAACACAG ACTCTGCAGAGATCCGGGGTCTCGAAGGT-3'*) and to correct c.3948insT mutation in exon 32 of *COL7A1*, ssDNA containing corrected *COL7A1* sequences in exon 32 was used (5'-*GATGGGCGTCCAGGCAGCCCTGGCCGCGCCGGGAATCCTGGGACCCCTGGA*

GCCCCTGGCCTAAAGGTGAGCAAGCCTTGTCCTGCAGGTCAGGGTGGGCGCT

GCCTGAGTGGGTGGGGTGGCTCCGACTGTT-3'). For gene editing, $2x10^6$ of single, patient-derived iPSCs harboring either the homozygous c.2470insG mutation in exon 19 of *COL7A* or the heterozygous c.2470insG mutation in exon 19 and c.3948insT mutation in exon 32 of *COL7A1* gene were electroporated with 15 sgRNA, 15ug ug of *SpyFi* Cas9, 10 ug of ssDNA repair donor template, and a plasmid encoding GFP using Amaxa Nucleofector 2 Device (Lonza) transfection system with Nucleofector Kit for human stem cells. The iPSCs were then recovered in E8 medium supplemented with RevitaCell Supplement for 48 hours after electroporation. 48 hours post-transfection, the GFP-positive iPSCs were collected via FACS, plated at a low density in 6-well plates, and cultured for 10 days until iPSC colonies were ready to be picked.

iPSC colony expansion. The emerging iPSC colonies were mechanically picked in a biological safety cabinet using a microscope. One half of the colony was plated onto a new vitronectin coated plate for further expansion and the second half was used for genomic DNA extractions. Genomic DNA was extracted using the QuickExtract DNA Extraction Solution (Epicenter) following the manufacturer's protocol.

Assessment of CRISPR/Cas9 activity by T7 endonuclease assay. 300–500 bp around the gene-edited locus were amplified by PCR using Herculase II (Agilent) and were column purified. The PCR was performed by using following primers: for c.2470insG mutation Fw-*CACTGACCTCCTCCTCCTCA*, Rev-*CAATCCTGGTCCTTTCTCCA* and for c.3947insT mutation Fw-*CTCTGAGGGGCCATTTCTCT*, Rev-*AAACAGAGCAGAGGGTGGTG*. PCR amplicons were rehybridized and treated with T7 endonuclease according to the manufacturer's directions (New England Biolabs). Digested DNA was separated on a

4–20% TBE polyacrylamide gel (BioRad) and imaged using SYBR Gold (Life Technologies).

Off-target analysis. Gene-edited homozygous and heterozygous RDEB iPSCs were tested for off-target editing events predicted for each gRNA by the Zhang laboratory CRISPR design tool (http://crispr.mit.edu) which also considers insertions or deletions in the guide RNA target sequence. The top five non-overlapping predicted off-target sites for each sgRNA were used. The region surrounding each off-target site was PCR-amplified, Sanger sequenced (GENEWIZ) and compared to the unedited cell line.

Feeder-free direct differentiation of iPSCs to keratinocytes (iKCs). Feeder-free iPSC-derived keratinocytes (iKCs) were generated with clinical applications in mind. iPSCs were maintained in E8 medium without a feeder layer. For keratinocyte differentiation, small clumps of iPSCs were subcultured onto vitronectin-coated plates. At 70% confluence the iPSCs monolayer culture were incubated in E8 medium supplemented with 1 μM all-trans RA (Sigma) and 10 ng/mL BMP4 (R&D Systems) for 7 days, as previously described (4). At day 7, the medium was switched to Defined Keratinocyte Serum-Free Medium (Gibco), and iPSCs were maintained in culture without passaging for 12, 30 or 60 days. After 4 passages, the iPSC-derived keratinocytes were subjected to *in vitro* and *ex vivo* functional analysis.

Feeder-free direct differentiation of iPSC to fibroblasts (iFBs). Feeder-free iPSC-derived fibroblasts were also generated with clinical applications in mind. To generate feeder-free iPSC-derived fibroblasts, iPSCs were harvested to form embryoid bodies (EB) using the AggreWell system (StemCell Technologies) at a size of 1000 cells/EB in E8 medium. 24 hr later, the EBs were harvested and plated onto 0.1% gelatin coated tissue culture dishes and cultured in DMEM with 20% FBS and 0.3 mM ascorbic acid

(Sigma-Aldrich) for 10 days. The medium was then switched to DMEM with 20% FBS without ascorbic acid for 10 days. At this point medium was switched to DMEM with 10% FBS and the cells were passaged once confluent and until spindle-shaped cells were prevalent.

FACS Analysis. The differentiated keratinocytes and fibroblasts from iPSCs were enzymatically collected and fixed with 4% PFA/PBS for 5 min at room temperature. After being washed with OBS, cells were blocked and permeabilized in PBS with 10% goat serum and 0.1% saponin (Sigma-Aldrich). Primary antibodies were incubated for 1 hour in PBS with 1% goat serum and 0.1% saponin. Normal IgG and no primary antibody were used as negative controls. After the secondary antibody conjugated with fluorescence protein was reacted, cells were analyzed on BD LSRII Cell Analyzer by use of BD FACSD via software (BD Biosciences). All primary antibodies used in this study are listed in Table 1 in SI.

Western blot analysis. iPSC-derived Fibroblasts were grown for 48 hours in the presence of 20 ng/ml transforming growth factor-b2 (Abcam) and 50 mg/ml ascorbic acid as previously reported(9). The medium was collected and directly analyzed by western blot analysis with the polyclonal rabbit LH7.2 antibody to human C7 (kindly provided by Dr. A. Nystrom). Cell lysates were extracted by centrifugation and the supernatant was precipitated. Proteins were collected by centrifugation at 14,000 revolutions per minute. The protein pellet was dissolved in 1 laemmli buffer and probed using the RC1 polyclonal antibody against human C7. Total protein content was determined using the micro-Lowry method DC Protein assay kit (Bio-Rad). Protein samples were loaded and separated on 3-8% gradient sodium dodecyl sulfate-polyacrylamide gels under reducing conditions. Secondary antibodies were horse anti-rabbit IgG antibodies, conjugated to horseradish peroxidase (Cell Signalling

Technology). Two different experiments were performed in duplicate. Signals were revealed with ECL-plus reagent (Thermo Scientific) using ImageLab software (Bio-Rad).

Trypsin assay. To investigate the folding of recombinant C7 produced in gene-edited and differentiated from EB iPSC fibroblasts, we analyzed the stability of secreted recombinant C7 by measuring its resistance to limited trypsin digestion at different temperatures, as described previously [41]. Briefly, the culture medium of gene-edited fibroblasts and wild type fibroblasts (differentiated from iPSCs) were collected on ice and centrifuged at 400 g for 5 minutes at 4°C. Proteolysis with pepsin and trypsin were performed as described earlier (9, 10). Medium without protease inhibitors was incubated at 38°C using a thermocycler (BioRad). Quantification was performed as previously reported (11).

3D human skin equivalents. 3D human skin equivalents (HSEs) were generated similar to the method described previously using the iFBs and iKCs (12, 13). After maintaining the HSEs in epidermalization medium for 7 days, they were moved to a new plate to expose them to an air-liquid interface in cornification medium. After 7 days in cornification medium, they were harvested for grafting onto mice.

Engraftment of iPSC derived 3D skin constructs on mice. All experimental animal protocols were approved by the Institutional Animal Care and Use Committee at Columbia University Medical Center, and the grafting was performed as previously reported (14). 0.8 cm of skin was removed from the dorsal anterior-posterior midline surface of 8–10-week-old NU(NCR)-Fon1nu nude mice (Charles River, Wilmington, MA) using the pinch-cutting technique. The 3D skin constructs were placed on the recipient and secured with 4 sutures (7-0 Nylon) in a simple interrupted pattern around the edge of the graft. To protect the equivalent, we used a method described for the

establishment of a humanized skin (14, 15) whereby the skin removed from the mouse was freeze-thawed 3 times by placing in DMEM, then inserting into liquid nitrogen prior to warming in a beaker of water. This decellularized and devitalized the skin, which was placed back over the skin construct on the mouse. The decellularized skin was sutured in place with interrupted sutures. A bandage was then wrapped around the mice (an OpSite Flexifix Transparent Film) to hold the graft in place. The mice were euthanized after 2 months for analyses. The constructs were harvested for IF and TEM analyses.

Immunostaining and Imaging. For immunostaining analyses, samples were either embedded in paraffin wax or cryopreserved in OCT solution. Formalin (10%) fixed, paraffin wax-embedded tissues were cut (15 μm) onto poly-L-lysine-coated slides, dried overnight at 55°C, dewaxed in xylene, and rehydrated through a graduated ethanol series (100%, 95%, 70%) to distilled water (dH2O). Samples were incubated with primary antibodies overnight at 4°C. After washing with PBS, samples were incubated with fluorophore-conjugated secondary antibodies (Invitrogen) for 2 hours at RT. Slides were covered with cover-slips using mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vectashield) and examined using a Zeiss LSM 5 Exciter confocal laser scanning microscope. All primary antibodies used in this study are listed in Table 1 in SI.

Haematoxylin and Eosin Staining (H&E). Dewaxed 15 μm sections were stained with Mayers Haematoxylin (Sigma) at RT for 3 minutes. Blue staining was performed by rinsing in tap water while differentiation was achieved by rinsing in 1% acid ethanol. Counterstaining was performed by rinsing with eosin (Sigma) for 30 seconds and dehydration was performed by sequential washing with 95% ethanol, 100% ethanol, and Histo-Clear (National Diagnostics). Slides were covered with cover-slips with DPX

(Agar Scientific) and examined by light microscopy using a Zeiss Axioplan 2 microscope.

Transmission electron microscopy (TEM). For ultrastructural analysis, tissue samples were cut to 2 mm^3 small pieces and immediately fixed in 4% glutaraldehyde in Sorensen's buffer, pH 7.4 and stored at 4°C in the fixative. The ultrastructural analysis was performed at the Division of Dermatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan.

SI Figures

Figure S1

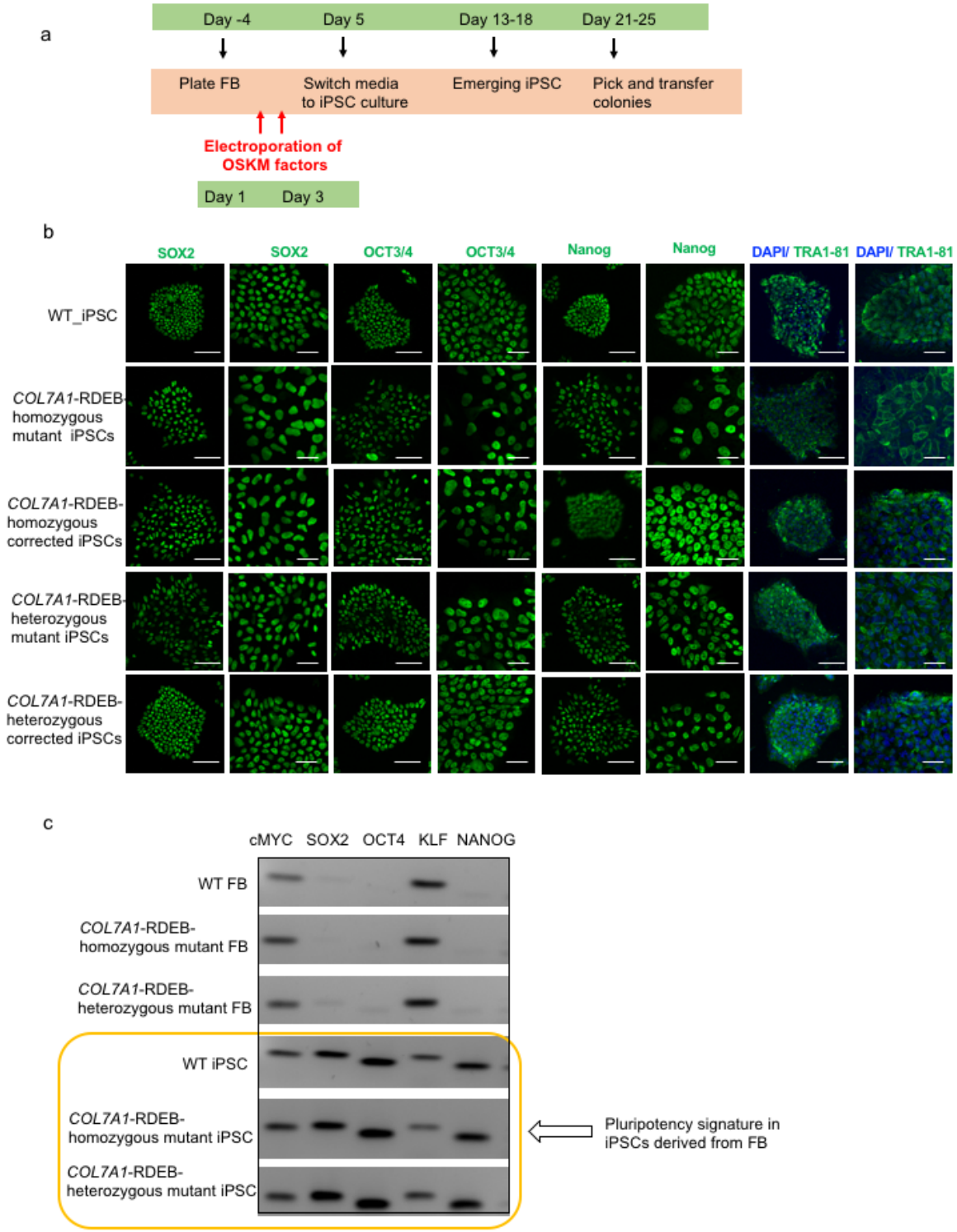


Figure S1. Stem cell signature of iPSCs generated from fibroblasts. (**a**) Timeline for reprogramming fibroblasts to iPSCs using integration-free episomal plasmids. $2x10^6$/ml fibroblasts were seeded into 10 cm^2 culture dishes and used in 4 days for electroporation. Fibroblasts were cultured in complete DMEM medium with 10% FBS for 4 days. On day 0 and 3, cells were electroporated with four reprogramming vectors (pCXLE-hOCT3/4-shp53-F: OCT3/4 and p53 shRNA, pCXLE-hSK: SOX2 and KLF4, pCXLE-hUL: $_L$-Myc and LIN28, pCXWB-EBNA1: EBNA1). At day 13, the cells show morphological changes indicative of reprogramming, and were plated after day 21 on vitronectin-coated plates under feeder-free conditions. The cells were cultured with Essential 8 medium for iPSC cells. Bar=10um. (**b**) The morphology of the iPSCs derived from fibroblasts and the expression of stem cell markers, including OCT3/4, SOX2, Nanog, and TRA1-81 were confirmed by immunofluorescence staining. Bar=10um. (**c**) Pluripotency signature in WT and RDEB iPSCs derived from FB was confirmed by RT-PCR analysis.

Figure S2

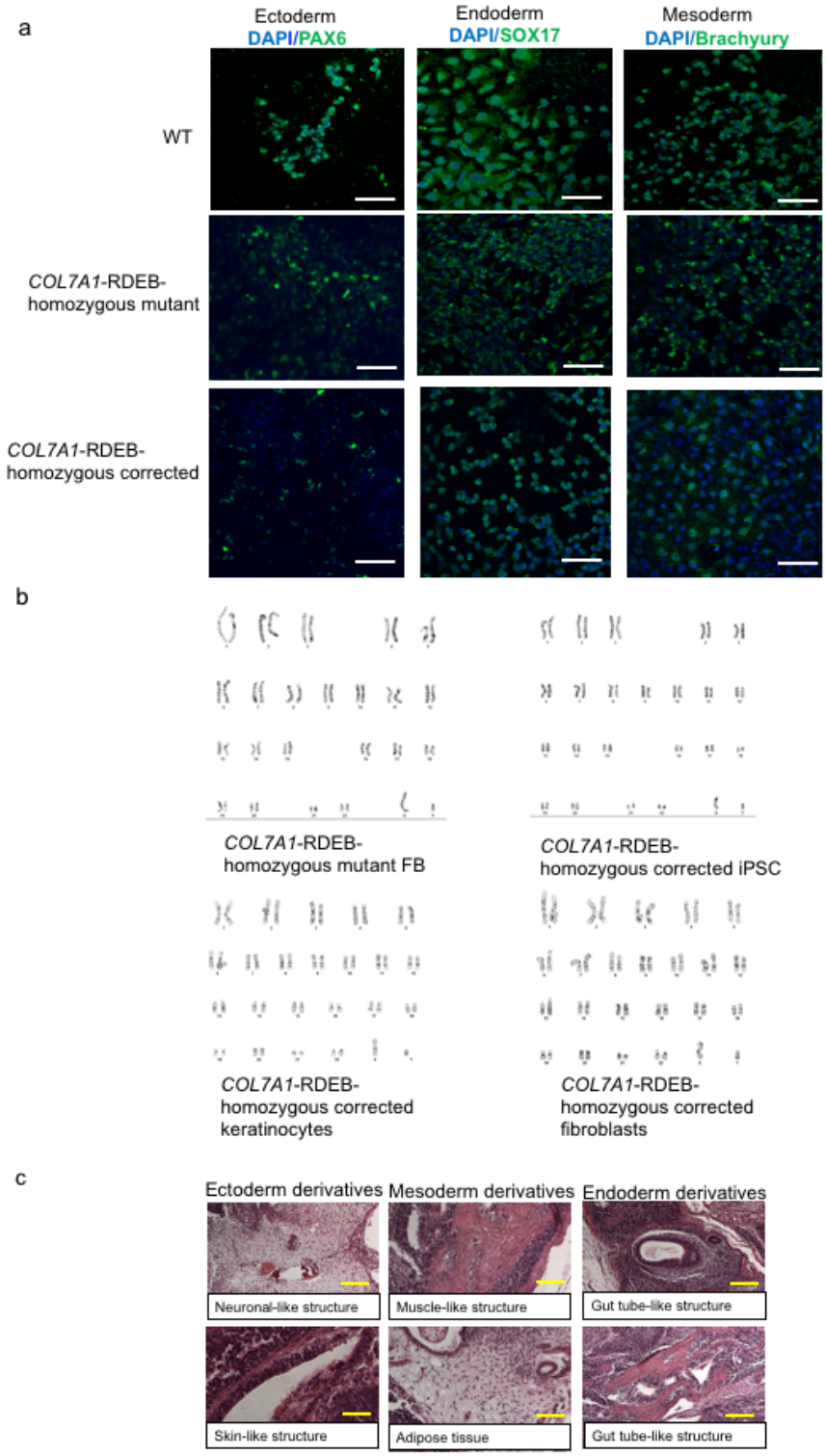

a
Ectoderm
DAPI/PAX6
Endoderm
DAPI/SOX17
Mesoderm
DAPI/Brachyury
WT
COL7A1-RDEB-homozygous mutant
COL7A1-RDEB-homozygous corrected
b
COL7A1-RDEB-homozygous mutant FB
COL7A1-RDEB-homozygous corrected iPSC
COL7A1-RDEB-homozygous corrected keratinocytes
COL7A1-RDEB-homozygous corrected fibroblasts
c
Ectoderm derivatives
Mesoderm derivatives
Endoderm derivatives
Neuronal-like structure
Muscle-like structure
Gut tube-like structure
Skin-like structure
Adipose tissue
Gut tube-like structure

Figure S2. Characterization of iPSCs. (**a**) We confirmed the differentiation capabilities of iPSC lines into all three germ layers by embryoid body formation *in vitro*. Pax6, SOX17, and Brachyury indicate ectodermal, endodermal, and mesodermal differentiation, respectively. (Scale bars: 200 um). (**b**) Karyotype analysis of *COL7A1*-RDEB-homozygous mutant iPSCs and homozygous gene-corrected keratinocytes and fibroblasts were similar to patients' FBs before reprogramming. No aneuploidy was observed. (**c**) The *in vivo* differentiation of iPSCs derived from RDEB patients FBs were shown by teratoma formation after intradermal injection of iPSCs into nude mice, indicating that the iPSCs had the capacity to differentiate into all three germ layers (Scale bars: 200 um).

Figure S3

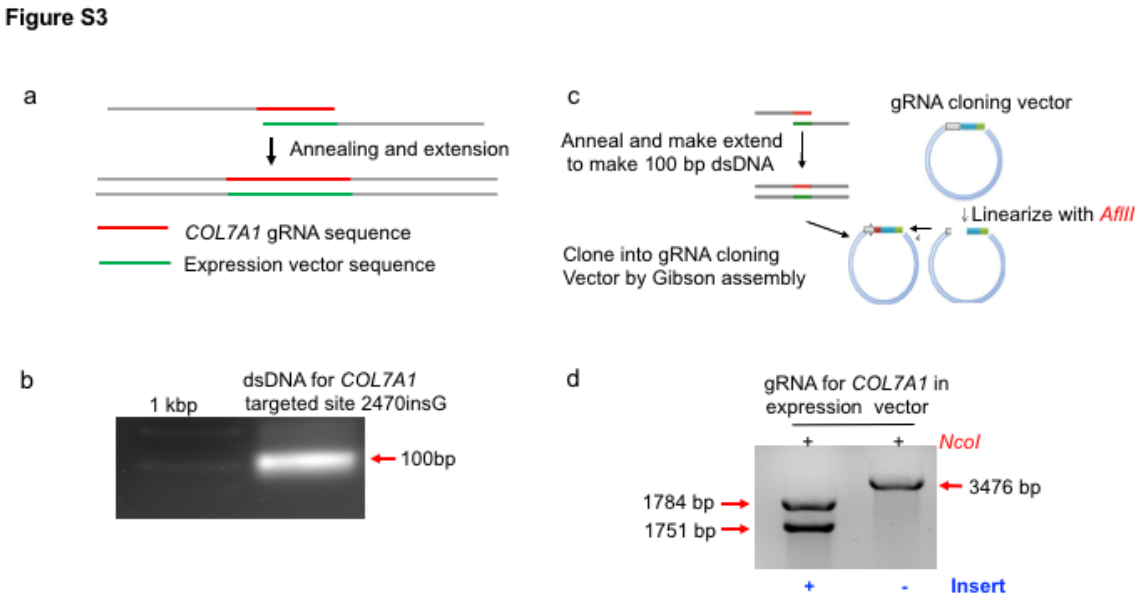


Figure S3. Design and synthesis of gRNA expression construct for 2470insG mutation in exon 19 of *COL7A1* gene. (**a**) Incorporation of 19 bp of the selected *COL7A1* target sequence into 60mer oligonucleotides which have a 20 nt complementary region with each other. Complementary sequences are highlighted in green and red. (**b**) 100 bp fully double-stranded DNA product for *COL7A1* was extended and annealed using the Phusion polymerase and verified by gel electrophoresis. (**c**) The 100 bp dsDNA fragment was incorporated into the gRNA expression vector using isothermal assembly known as Gibson assembly (7). (**d**) Restriction digestion of gRNA expression vector with incorporated *COL7A1* targeted sequence with *NcoI* confirmed the successful Gibson assembly reaction.

Figure S4

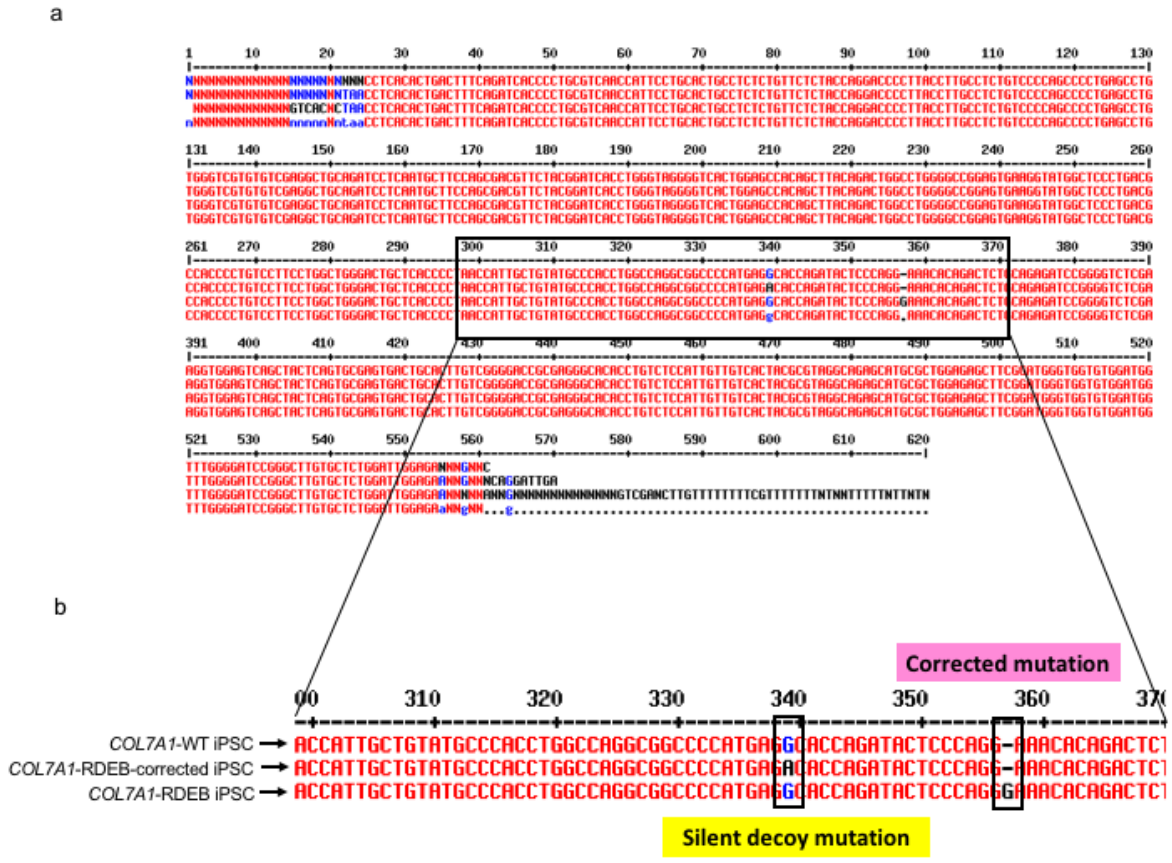


Figure S4. Sequence alignment. Comparison of sequence alignment of gene-corrected *COL7A1*-RDEB DNA with the parental *COL7A1*-RDEB mutated DNA and *COL7A1*-WT control DNA illustrates the gene correction and proper integrity of the genomic sequence flanking the modified exon after HDR. Note that one extra silent nt change was intentionally-included in the repair donor template to create a decoy site in order to prevent Cas9 from cleaving after the mutation has been corrected.

Figure S5

a

		No	Mismatch	Loci	T7 test
On-target	CT GGC CAG GCG GCC CCA TGAGG(PAM)	#1			
Off-target	CGTGGCCAGGCA GCCCCAGGAGG	1	3	Chr14:105561644-105561666	negative
Off-target	CCTGCCCGGGCAGCCCCATGAGG	2	3	Chr6:150116691-150116713	negative
Off-target	CCTGCCCAGCCGGCCCCATGTGG	3	3	Chr8:41435262-41435284	negative
Off-target	CCTGGGCAGTCAGCCCCATGAGG	4	3	Chr22:27618278-27618300	negative
Off-target	CCTGGCCAGGAGGCCCCAGGAGG	5	2	Chr2:120449610-120449632	negative
On-target	CTGCAGAGTCTGTGTTTCCCTGG(PAM)	#2			
Off-target	CTGCAGAGGCTGTGTTTCTCTGG	6	2	ChrX:48967756-48967778	negative
Off-target	CTGCAGAGTCTGAGTTTCTCTGG	7	2	Chr18:52734568-52734590	negative
Off-target	CTGCAGAGTCTGGGTTTCCCAGG	8	2	Chr1:39686793-39686815	negative
Off-target	CTGCAGAGTCTGTGTTTCCTGGG	9	2	Chr3:48588741-48588763	negative
Off-target	CTGCAGGGTCTGTGTTGTCCCTGG	10	1	Chr14:51303283-51303306	negative
On-target	TGGCCGCGCCGGGAATCCT TGGG (PAM)	#3			
Off-target	TGGCCGCGCCGGGAATCCTGGGA	11	2	Chr3:48585059-48585081	negative
Off-target	^GGCCGCGCCGGGAATCCTGGGA	12	2	Chr3:48585059-48585080	negative
Off-target	TGG^CGCGCCGGGAGTCCCTGGG	13	2	Chr2:112764961-112764982	negative
Off-target	TGGCCGCGCCGGGAA^TCCTGGG	14	2	Chr3:48585060-48585081	negative
Off-target	TGGCGACGCCGGGAAT^CTTGGG	15	2	Chr6:158062359-158062380	negative

b

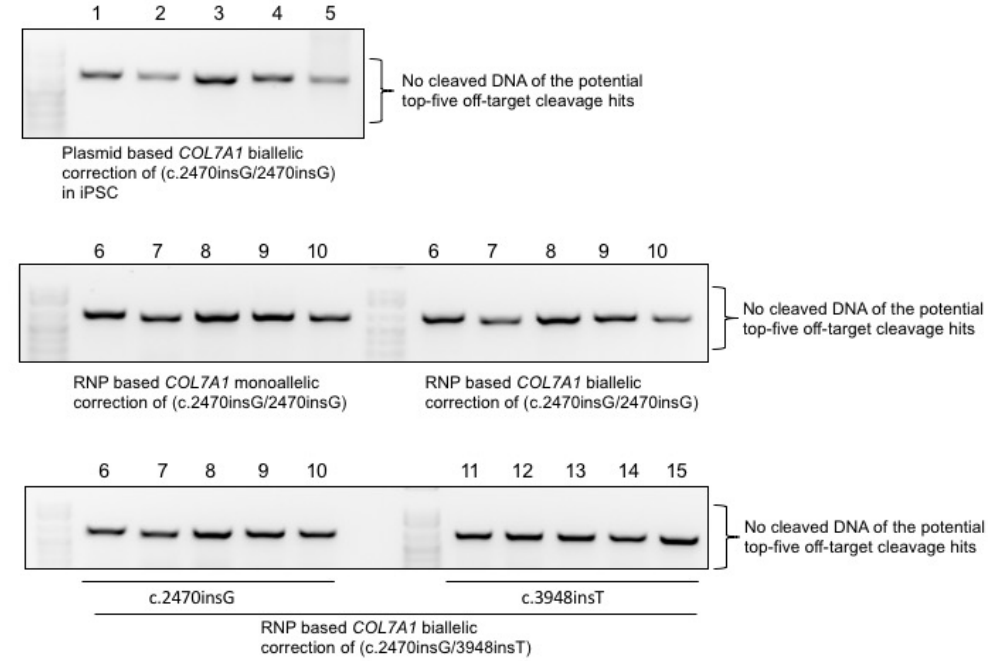
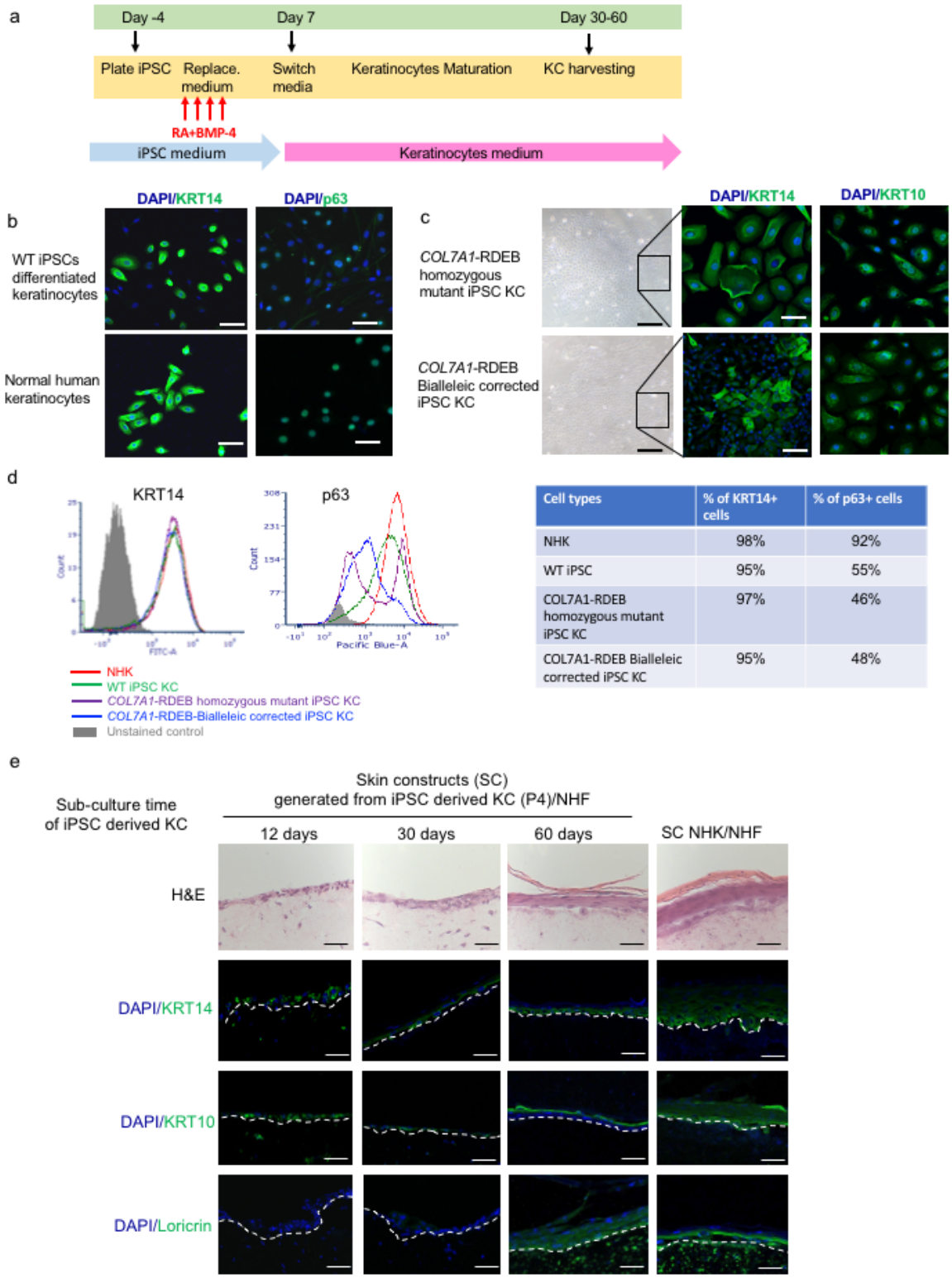


Figure S5. Off-target site analysis. (**a**) List of the five most similar off-target sites predicted using the COSMID CRISPR design tool, each for 2470insG gRNA (#1) used for homozygous c.2470insG mutations by plasmid strategy; 2470ins gRNA (#2) used for homozygous and heterozygous c.2470insG mutation by RNP strategy; 3948insT gRNA (#3) used for heterozygous c.3948insT mutation by RNP strategy. Red bases indicate sequence differences from the target sequence. (**b**) The top-five off target cleavage hits were amplified and the PCR products were analyzed by T7 test and Sanger sequencing. A negative T7 test demonstrates that no off-target indels were identified.

Figure S6



Cell types	% of KRT14+ cells	% of p63+ cells
NHK	98%	92%
WT iPSC	95%	55%
COL7A1-RDEB homozygous mutant iPSC KC	97%	46%
COL7A1-RDEB Bialleleic corrected iPSC KC	95%	48%

Figure S6. Directed differentiation of iPSCs into keratinocytes under feeder-free conditions. (**a**) Schematic representation of the differentiation strategy for generating KCs from iPSCs. 1uM retinoic acid (RA) to promote ectodermal fate and bone morphogenetic protein 4 (BMP4) to block neural fate were added daily for 6 days as previously reported (4). The cells were kept without passaging for more than 30 days to promote full maturation after differentiation. (**b**) Immunofluorescence (IF) staining of iPSC-derived KCs for keratin 14 and p63 at 60 days of maturation, compared to NHKs. (**c**) Immunofluorescence (IF) staining of iPSC-derived *COL7A1*-RDEB mutant and corrected iKCs for keratin 14 and keratin 10 expression. (**d**) FACS analysis of keratin 14 and p63 expression at 60 days of maturation in iPSC-derived keratinocytes lines. (**e**) H&E of 3D skin constructs made of 12, 30, and 60 day-matured iPSC-KCs and normal human fibroblasts (NHFs) compared to 3D skin constructs produced from NHKs and NHFs demonstrated that 60 days are needed to mature functional keratinocytes, which can then proliferate and build a stratified epidermis. IF staining for keratin 14, keratin 10, and loricrin are demonstrated (Bar=10um).

Figure S7

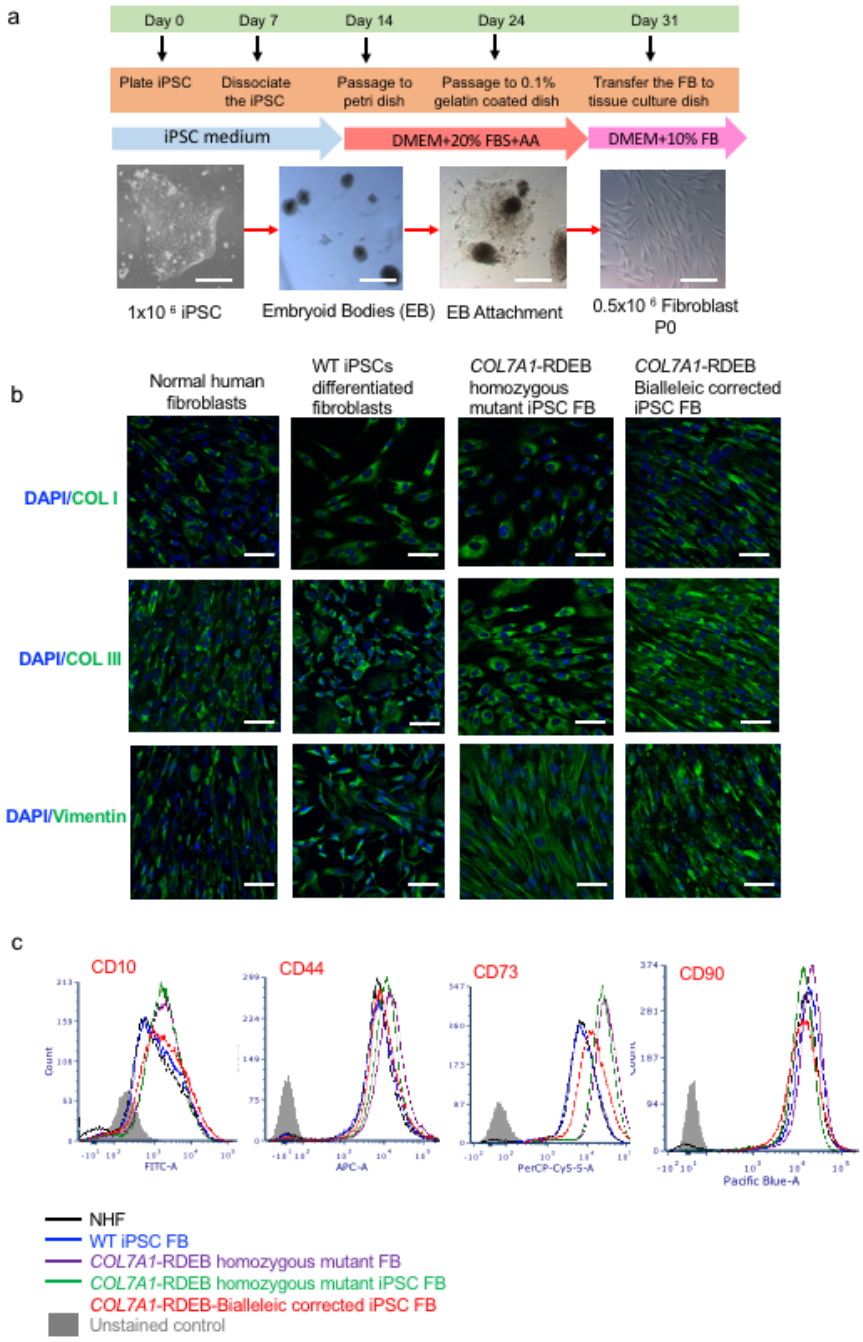

a
Day 0
Day 7
Day 14
Day 24
Day 31
Plate iPSC
Dissociate the iPSC
Passage to petri dish
Passage to 0.1% gelatin coated dish
Transfer the FB to tissue culture dish
iPSC medium
DMEM+20% FBS+AA
DMEM+10% FB
1x10 6 iPSC
Embryoid Bodies (EB)
EB Attachment
0.5x10 6 Fibroblast P0
b
Normal human fibroblasts
WT iPSCs differentiated fibroblasts
COL7A1-RDEB homozygous mutant iPSC FB
COL7A1-RDEB Bialleleic corrected iPSC FB
DAPI/COL I
DAPI/COL III
DAPI/Vimentin
c
CD10
CD44
CD73
CD90
FITC-A
APC-A
PerCP-Cy5-5-A
Pacific Blue-A
NHF
WT iPSC FB
COL7A1-RDEB homozygous mutant FB
COL7A1-RDEB homozygous mutant iPSC FB
COL7A1-RDEB-Bialleleic corrected iPSC FB
Unstained control

Figure S7. **Directed differentiation and characterization of iPSCs into fibroblasts**. (**a**) Schematic representation of the differentiation strategy for generating fibroblasts from iPSCs under feeder-free conditions. Representative images demonstrate cells growing out from attached embryoid bodies made in medium supplemented with ascorbic acid (AA) and TGFβ-2, which were repeatedly split to obtain a fibroblast-like cell population(13). Morphology of spindle-shaped iPSC-derived fibroblasts was observed after passage 1 (P1). Bar=10um. (**b**) Immunofluorescence staining of COL I, COL III, and vimentin of iPSC-derived fibroblasts and normal human fibroblasts. (**c**) Cell surface marker expression in iPSC-derived fibroblasts. FACS analysis showed that the cell surface marker profile of iPSC-derived fibroblasts was similar to normal human fibroblasts.

Figure S8

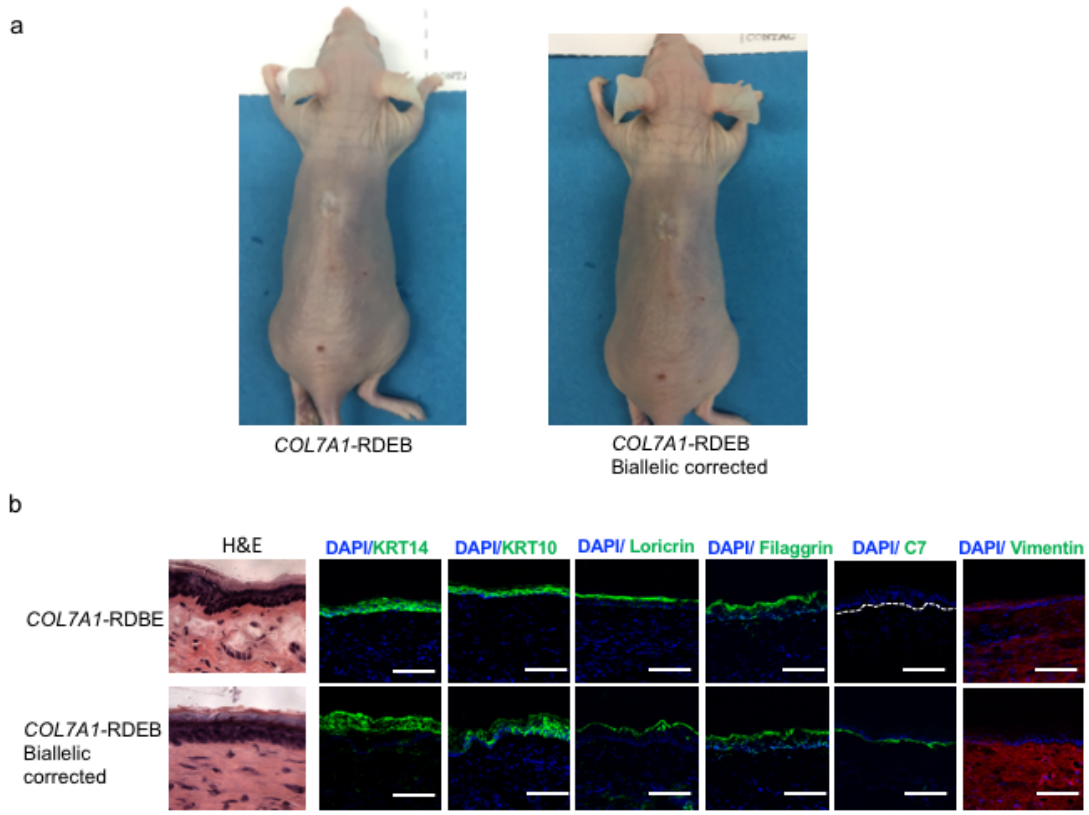


Figure S8. Healthy skin appearance and C7 deposition in 9 months old mice grafted with iPSC-derived gene-corrected skin equivalents. (**a**) Pictures of mice grafted with skin equivalents made from *COL7A1*-RDEB mutant iPSC-derived KCs and FBs and *COL7A1*-RDEB biallelic-corrected iPSC-derived KCs and FBs. (**b**) H&E staining revealed normal epidermal and dermal morphology. C7 deposition is demonstrated by immunofluorescence (IF) staining (green signal), 9 months after grafting using LH7.2 antibody. Additional IF staining were performed for keratin 14, keratin 10, loricrin, fillagrin, and vimentin on corrected, mutant, and wild-type xenografts.

Table S1 Primary antibodies used in this study

Primary Antibody	Clone or catalog number	Company
Anti-Brachyury	6C12	Abcam
Anti-CD10	HI10a	BD Biosciences
Anti-CD44	G44-26	BD Biosciences
Anti-CD73	AD2	BD Biosciences
Anti-CD90	5E10	BD Biosciences
Anti-Collagen I	ab6308	Abcam
Anti-Collagen III	70R-CR014	Fitzgerald
Anti-Fillagrin	Poly 19058	BioLegend
Anti-Keratin 10	PRB-159P	BioLegend
Anti-Keratin 14	PRB-155P	BioLegend
Anti-Loricrin	PRB-154P	Covance
Anti-Nanog	EPR2027	Abcam
Anti-Oct-3/4	C-10; sc-5279	Santa Cruz Biotechnology
Anti-p63	H-137; sc-8343	Santa Cruz Biotechnology
Anti-PAX6	ab154253	Abcam
Anti-SOX17	Ab155402	Abcam
Anti-SOX2	Y-17; sc-17320	Santa Cruz Biotechnology
Anti-TRA1-81	MAB4381	Millipore
Anti-Type VII collagen	Clone LH7.2	Millipore
Anti-Vimentin	V9; sc6260	Santa Cruz Biotechnology

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