

Science

26 November 2014

Translational Medicine

Editor's Summary

Patient-Specific Stem Cell Therapy for Rare Skin Disease

Recessive dystrophic epidermolysis bullosa (RDEB) is characterized by a mutation in the *COL7A1* gene, which causes severe skin fragility and blistering. Although rare, patients with RDEB spend their lives in pain, fearful of even the slightest amount of friction. There are no cures for this disease, but cell therapy represents a viable option, as demonstrated by Sebastiano *et al.* in this issue. The authors took skin biopsies from three adult patients with RDEB and generated induced pluripotent stem cells, or iPSCs, from the keratinocytes (skin cells) and fibroblasts present in the tissue. The *COL7A1* mutation in the iPSCs was corrected using a new adeno-associated viral approach. The genetically repaired iPSCs were screened to make sure they did not have any genes associated with squamous cell carcinoma—a cancer common to RDEB patients—and were then differentiated into keratinocytes that expressed full-length wild-type collagen VII protein. In vitro and in vivo in mice, these "corrected" keratinocytes were able to form sheets of skin with a defined layer of collagen VII. Although the skin grafts only lasted for 3 weeks, and further testing is needed in a disease model, the ability to correct and bank a patient's own cells and to select "clean" iPSCs represents an important step forward in devising a treatment for those affected by RDEB.

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SKIN DISEASE

Human *COL7A1*-corrected induced pluripotent stem cells for the treatment of recessive dystrophic epidermolysis bullosa

Vittorio Sebastiano,^{1,2*} Hanson Hui Zhen,^{3*} Bahareh Haddad Derafshi,¹ Elizaveta Bashkirova,³ Sandra P. Melo,³ Pei Wang,^{4†} Thomas L. Leung,⁴ Zurab Siprashvili,³ Andrea Tichy,³ Jiang Li,³ Mohammed Ameen,³ John Hawkins,¹ Susie Lee,³ Lingjie Li,³ Aaron Schwertschcow,⁵ Gerhard Bauer,⁵ Leszek Lisowski,^{6‡} Mark A. Kay,⁶ Seung K. Kim,⁴ Alfred T. Lane,³ Marius Wernig,^{1§} Anthony E. Oro^{3§}

Patients with recessive dystrophic epidermolysis bullosa (RDEB) lack functional type VII collagen owing to mutations in the gene *COL7A1* and suffer severe blistering and chronic wounds that ultimately lead to infection and development of lethal squamous cell carcinoma. The discovery of induced pluripotent stem cells (iPSCs) and the ability to edit the genome bring the possibility to provide definitive genetic therapy through corrected autologous tissues. We generated patient-derived *COL7A1*-corrected epithelial keratinocyte sheets for autologous grafting. We demonstrate the utility of sequential reprogramming and adenovirus-associated viral genome editing to generate corrected iPSC banks. iPSC-derived keratinocytes were produced with minimal heterogeneity, and these cells secreted wild-type type VII collagen, resulting in stratified epidermis in vitro in organotypic cultures and in vivo in mice. Sequencing of corrected cell lines before tissue formation revealed heterogeneity of cancer-predisposing mutations, allowing us to select *COL7A1*-corrected banks with minimal mutational burden for downstream epidermis production. Our results provide a clinical platform to use iPSCs in the treatment of debilitating genodermatoses, such as RDEB.

INTRODUCTION

Epidermolysis bullosa (EB) represents a group of debilitating inherited skin disorders in which blisters develop after relatively minor trauma to the skin. EB results from mutations in at least 18 different genes, mostly encoding structural components of the basement membrane zone or within basal keratinocytes (1). Although EB is a rare disease (it has an incidence of 11 per million live births in the United States) (2), the worldwide estimated incidence is about half a million individuals. One particularly disabling form, autosomal recessive dystrophic epidermolysis bullosa (RDEB), derives from mutations in the *COL7A1* locus, a gene that encodes for type VII collagen, the main component of the anchoring fibrils that tether the epidermis to the dermal tissue underneath. RDEB patients suffer profound skin fragility, delayed wound healing, and persistent erosions, with longer-term complications of scarring and increased incidence of malignancy (3).

Among the most effective attempts to develop a therapy for RDEB are the genetic engineering approaches that make use of both viral and nonviral vectors to efficiently transfer the *COL7A1* complementary DNA into primary patient keratinocytes with a concurrent phenotypic correction of the defect upon transplantation (4–8). This includes the

recent successful trial by our group to generate *COL7A1*-expressing retrovirally infected human epithelial sheets (9). Each of these approaches displays shortcomings associated with limited efficacy or safety risks. None of the approaches addressed the chronic wounding and severe depletion or exhaustion of epidermal stem cells in RDEB patients. Such depletion represents a key roadblock in somatic gene therapy efforts owing to the paucity of donor cells and potential for transformation from accumulated mutational load in remaining stem cells.

The generation of induced pluripotent stem cells (iPSCs) from human cells in 2007 was an important breakthrough for the field of regenerative medicine (10, 11). In principle, iPSC-based approaches would overcome the limitations associated with previous approaches. They can be generated from any individual from various cell types, such as fibroblast or blood cells. Unlike somatic cells, iPSCs have a high proliferation potential without senescing over time. Furthermore, they are amenable to genetic manipulations, including homologous recombination (HR), which allows the in situ correction of the disease-causing mutation. This genetically defined repair approach avoids several safety risks associated with conventional vector-based gene therapy involving random integration such as nonphysiological gene expression and cancer formation. Although these prospects are exciting, several new hurdles are associated with iPSC technology. Questions arise about the safety of the reprogramming and gene targeting methodologies, which involve extended culture periods, differentiation efficiency, and quality of iPSC-derived cells (12). These questions need to be answered before translation of iPSC-based technologies to the clinic.

Here, we show that despite their magnitude, in principle, those hurdles can be overcome. We demonstrate that iPSCs can be derived from RDEB patients, using reagents qualified for good manufacturing procedures. High targeting efficiencies were achieved at the *COL7A1* locus in these cells to repair the disease-causing mutation. The repaired iPSCs

¹Institute for Stem Cell Biology and Regenerative Medicine, and Department of Pathology, Stanford University, Stanford, CA 94305, USA. ²Department of Obstetrics and Gynecology, Stanford University, Stanford, CA 94305, USA. ³Program in Epithelial Biology, Department of Dermatology, Stanford University, Stanford, CA 94305, USA. ⁴Department of Developmental Biology, Stanford University, Stanford, CA 94305, USA. ⁵Institute for Regenerative Cures, School of Medicine, University of California, Davis, Sacramento, CA 95817, USA. ⁶Departments of Pediatrics and Genetics, Stanford University, Stanford, CA 94305, USA.

*These authors contributed equally to this work.

†Present address: University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229, USA.

‡Present address: Salk Institute for Biological Studies, San Diego, CA 92037, USA.

§Corresponding author. E-mail: wernig@stanford.edu (M.W.); oro@stanford.edu (A.E.O.)

were differentiated into stratifying and graftable keratinocytes that produced wild-type type VII collagen. Detailed genomic characterization of donor cells, primary iPSCs, and corrected iPSCs revealed an unexpectedly high genetic heterogeneity of even clonal cell populations. Furthermore, we identified existing and newly introduced mutations in 13 known squamous cell carcinoma (SCC) predisposition genes, and by using type VII collagen-corrected, cancer mutation-free keratinocytes, we regenerated skin tissue in mice.

RESULTS

Generation of iPSCs from RDEB patients

The workflow of our study is shown in Fig. 1A. We obtained skin biopsies from three adult patients with RDEB (Fig. 1B). Patient-specific iPSCs [original iPSCs (o-iPSCs)] were generated from fibroblast and keratinocyte primary cultures, using an integrating but excisable lentiviral reprogramming method (L4F) as described previously (13, 14) (Fig. 1C). This method was chosen over plasmid, RNA, and/or small-molecule reprogramming methods owing to the ease in tracking genomic changes and reproducibility of iPSC generation. Multiple iPSC clones were derived from three of the recruited patients (designated AO1, AO2, and AO3) from both keratinocytes and fibroblasts (Fig. 1B). Southern blot analysis revealed only one to two proviral integrations per clone (Fig. 1D). All established clones expressed the transcription factors OCT4 and NANOG and the surface markers SSEA3 and TRA-1-60 at the protein level (Fig. 1E and fig. S1). Karyotype analysis, performed by G-banding between passages 15 and 20, revealed that at least one clone of iPSCs per patient exhibited a normal karyotype, which was used for further studies (Fig. 1, B and E, and fig. S1).

Pluripotency was assessed by teratoma assay and the ability to form in vivo cell derivatives of ectoderm, mesoderm, and endoderm lineages (Fig. 1E and fig. S1A). Confirming the ability to manufacture patient-specific iPSC lines, we established iPSCs from RDEB patients using reagents qualified per U.S. Food and Drug Administration (FDA) standards and under good manufacturing procedures (GMP) (fig. S2). This involved the use of certified mouse feeder cells, the production of the lentivirus under GMP conditions, and the derivation and expansion of iPSCs using certified materials and reagents (see Supplementary Materials and Methods and fig. S2).

Correction of *COL7A1* mutations in RDEB patient-derived iPSCs

Previous reports indicated spontaneous reversion of keratinocytes within the skin of some EB patients, leading to the ability to expand and graft revertant autologous keratinocytes (15, 16). Indeed, a companion paper in this issue demonstrates the usability of these donor cells for potential therapeutic purposes (17). However, spontaneous reversion remains rare and EB subtype-specific, requiring the need for a general genome editing protocol to repair mutations in most patients (Fig. 1A). First, we explored conventional targeting methods, given the potential safety concerns around engineered proteins with nuclease activity such as zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs), and CRISPR/Cas9. Our previous studies indicated that the use of single-positive selection and double-negative selection had high rates of HR (18).

We therefore generated a targeting vector with 8.8- and 4.4-kb arms covering 31 exons and a central neomycin selection cassette flanked

by diphtheria toxin and thymidine kinase negative selection cassettes (Fig. 2A). Targeting experiments were performed using two different patient lines (F1-2 and K3-4) with confirmed compound heterozygous, recessive (loss-of-function) mutations. Resistant clones were assayed for HR by Southern blot analysis (Fig. 2B) and confirmed by Sanger sequencing (Fig. 2C). In two independent experiments, we found that the targeting frequency of the *COL7A1* locus was 11 and 3% with a frequency of correctly targeted clones of 26 and 75% (Fig. 2G). Because the *COL7A1* locus is weakly expressed in iPSCs, these recombination rates are consistent with previous experiments indicating that traditional HR rates correlate with level of expression in human embryonic stem (hES) cells (19).

We compared the targeting efficiency of conventional targeting with that induced by the novel adeno-associated viral (AAV) variant (AAV-DJ) that we recently identified as having a high recombinogenic activity (20). AAV-DJ-mediated targeting (AT) has the advantage that AAVs have previously been used in human gene therapy trials, have low off-target rates, and are easily detectable. An AAV-DJ variant with 1.4-kb *COL7A1* targeting arms was used to infect RDEB iPSC lines (F1-1 and K3-1) (Fig. 2D). Southern blot analysis (Fig. 2E) and Sanger sequencing (Fig. 2F) of K3-1 and F1-1 revealed higher recombination frequencies than with conventional targeting (56% and 16%, respectively) with correctly targeted clones at respective frequencies of 6% and 100% (Fig. 2G).

Both targeting constructs used a selection marker flanked by loxP sites. We next looped out the selection cassette together with the integrated reprogramming factor cassettes by transient Cre expression. We used different loxP sequences for the reprogramming and for the correction cassette to ensure that only cis-recombination events could occur during looping out. Successful looping-out was confirmed by polymerase chain reaction analysis and Southern blot of patient iPSC clones (Fig. 2, B and E, and fig. S3, A and B). G-banding analysis (performed between passages 30 and 40) confirmed a normal karyotype of the corrected and looped-out clones (fig. S3C). Correctly targeted and mutation-repaired clones showed no random integrations of AAV, as shown by Southern blot analysis (Fig. 2, B and E). Because AAV-DJ-mediated HR rates were comparable to those of CRISPR/Cas9 (fig. S4) (21), and potentially safer and easier to deliver, we concluded that AAV-DJ-mediated genome editing would provide the ideal genome editing system for correction during therapeutic iPSC manufacturing.

Genomic characterization during iPSC manufacturing

Given the extended culture periods required to derive a therapeutic cell product from genetically corrected iPSCs, we sought to characterize the genetic variations of the iPSCs during cell manufacturing. We sequenced two sets of donor somatic cells, three original iPSC clones (o-iPSC), and three corrected and Cre-excised iPSC clones (c-iPSC) (Figs. 1A and 3A) using Complete Genomics whole-genome sequencing platform (average coverage of 40×). Ingenuity Variant Analysis (IVA) software applying filter criteria specified in Supplementary Materials and Methods were used to call genetic variants in the fibroblast- or keratinocyte-derived iPSCs by comparing to the hg19 reference genome.

Variants were then assembled and categorized into functional groups (Fig. 3, B and C). Most variants in each case were common germline variants seen in both donor cells and iPSCs constituting about 54% of the total variants in each line. Variants were found at various call confidence levels as defined by the Complete Genomics analysis pipeline suggesting large variation of allele frequencies, likely reflecting fractions of different sizes of the sequenced cell population. For all three lines,

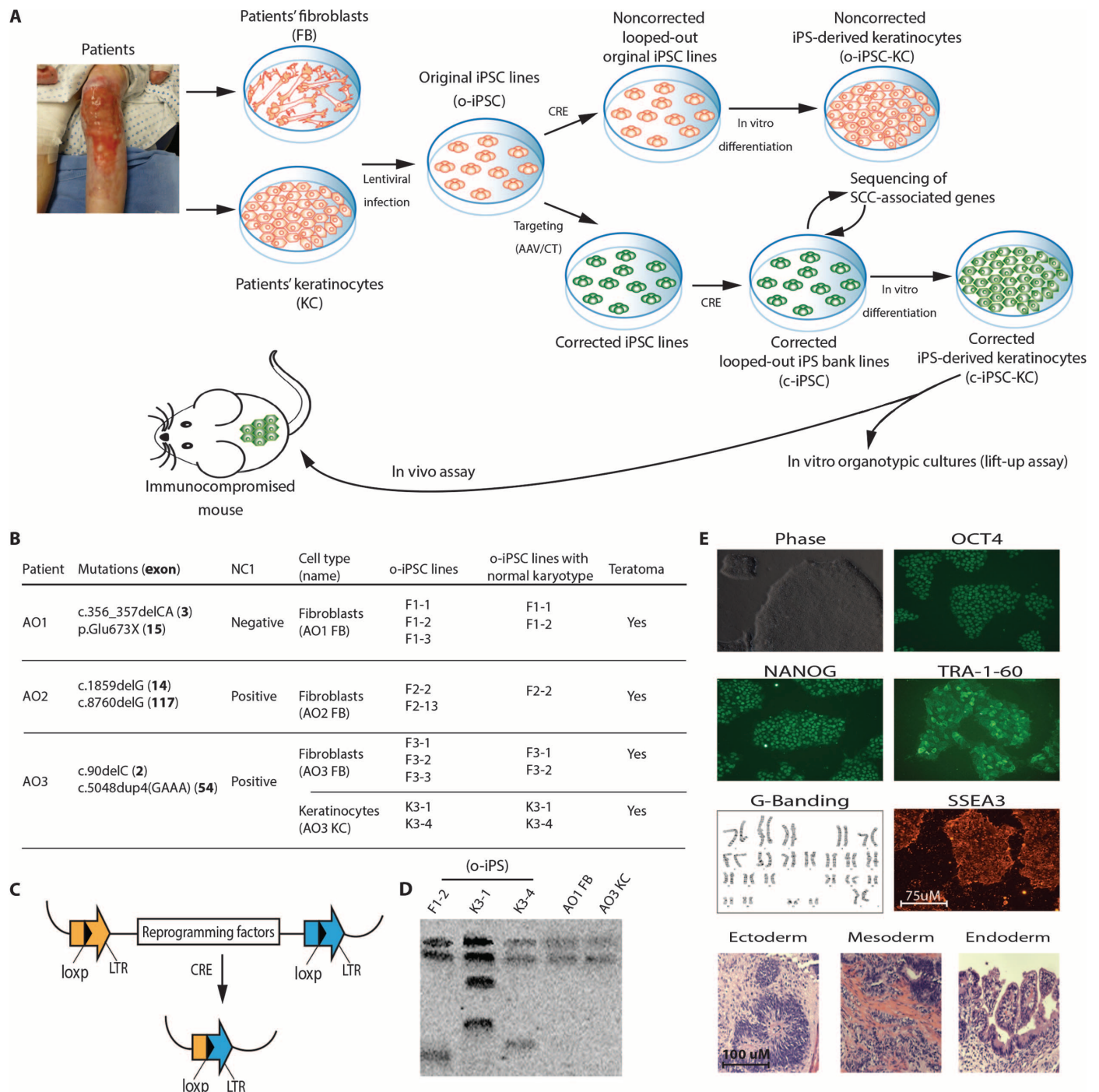


Fig. 1. Derivation and characterization of iPSCs from patients with RDEB. (A) Schematic overview of the protocol in this study. Fibroblasts and keratinocytes were derived and cultured from a skin biopsy, and iPSCs were established from both cell types. iPSCs were then either corrected in their *COL7A1* loci by AAV or conventional targeting and differentiated in vitro into keratinocytes (c-iPSC-KC), or left uncorrected and directly differentiated into keratinocytes (o-iPSC-KC). In vitro-derived keratinocytes (from corrected and noncorrected iPSCs) were used for organotypic cultures and for in vivo skin reconstitution assays in immunocompromised mice. Red cells are uncorrected; green cells are genetically corrected. (B) The patients for which iPSC clones were derived successfully. Information on patients' specific

recessive mutations in *COL7A1* locus is provided. NC1 is the immunogenic N-terminal domain of *COL7A1*. (C) Schematic representation of the lentiviral vector used to reprogram the patients' somatic cells. (D) Southern blot revealing the integration events of the lentiviral reprogramming cassette in somatic cells (fibroblasts and keratinocytes) and in the iPSC clones used in the study (F1-2, K3-1, and K3-4). (E) Characterization of iPSCs [clone F1-2 in (D)] revealing their bona fide undifferentiated and pluripotent state. Expression of a set of markers (OCT4, NANOG, TRA-1-60, and SSEA3) was identified by immunofluorescence. Normal karyotype was confirmed by G-banding. Pluripotency was assessed by teratoma formation and differentiation into cell derivatives of ectoderm, mesoderm, and endoderm.

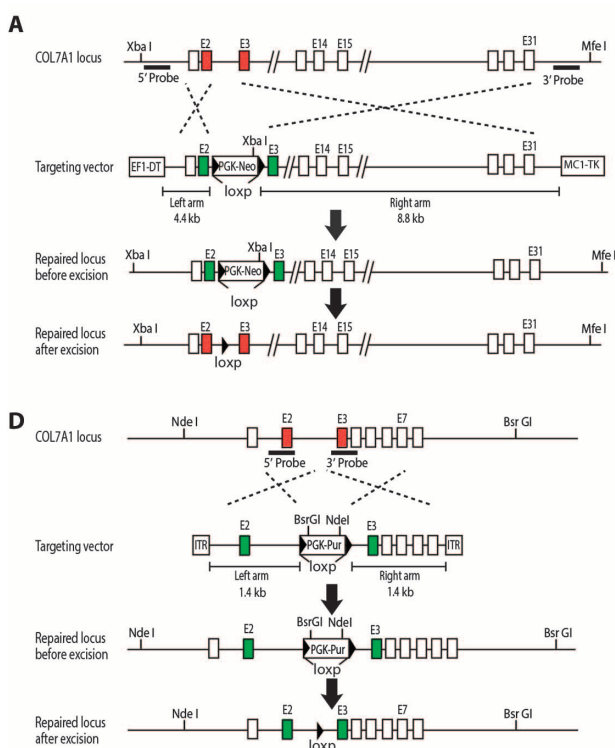


Fig. 2. HR-mediated correction of mutations in the *COL7A1* locus of RDEB-derived iPSCs. (A to C) Repairing the *COL7A1* locus by conventional targeting (A), with double-negative selection and positive selection by diphtheria toxin (DT). TK, thymidine kinase; Neo, neomycin resistance; PGK, phosphoglycerate kinase I promoter. Exons carrying the mutations are in red; wild-type exons are in green. Enzymes used for Southern blot analysis as well as the location of the probes, represented by black bars, are indicated. (B) Southern blot analysis of representative neomycin-resistant clones obtained in corrected iPSCs (c-iPSC) after conventional targeting (CT). KC, keratinocyte; FB, fibroblast. (C) DNA Sanger sequencing of mutant and targeted iPSCs. Mutation is shown as double peaks in the pre-correction sample, denoting the heterozygous nature of the mutations. (D to F) Repairing the *COL7A1* locus by HR. (D) AAV-mediated targeting using puromycin (Pur) selection with similar coloring as in (A). (E) Southern blot analysis of representative puromycin-resistant clones. (F) DNA Sanger sequencing showing mutant and corrected sequences, as in (C). (G) Comparison of conventional and AAV-mediated targeting methods.

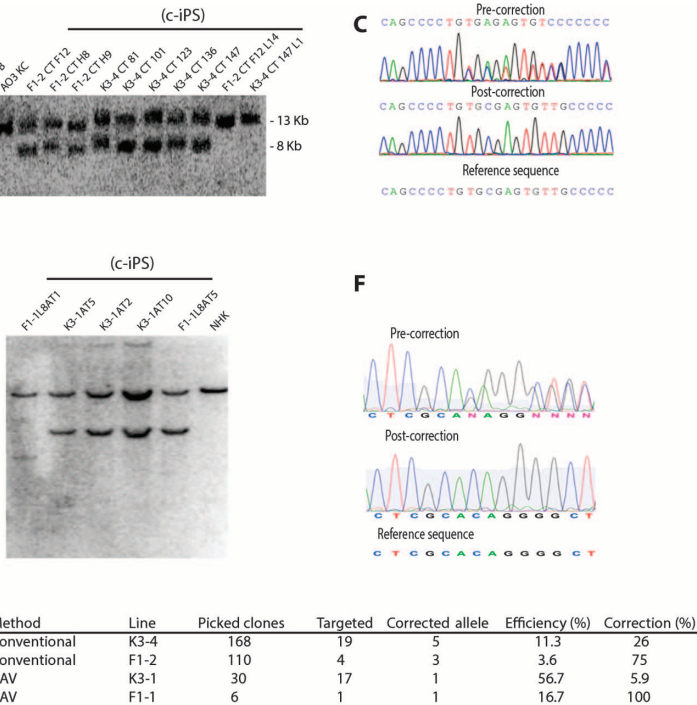
variants that occurred in original iPSCs constituted a small fraction of the variants seen in the donor cell culture and also contained additional variants not previously present. Moreover, none of the called variants overlapped between the three different lines (table S1). This pattern is consistent with random loss and gain of variants and not with continued selective pressure (Fig. 3B).

To substantiate the randomness of the variation during cell culture, we examined the Gene Ontology (GO) terms for the non-germline variants seen in the c-iPSCs. In each case, no GO term reached statistical significance (Fig. 3C). No particular biological process appeared to be driving the genetic variation in cell culture as seen in whole-genome sequencing.

Identification of SCC-predisposing mutations

Previous epidemiological studies indicate that patients with the severe generalized variant of RDEB that survive into late adulthood are at heightened risk for invasive SCC, with 55% dying from SCC by age 40 (3). Because of the potential risk of SCC-associated mutations in the donor keratinocytes and fibroblasts, we evaluated whether SCC-associated genes were selected in the creation of the corrected iPSCs and whether we could select lines that had reduced numbers of tumor-associated mutations.

We created custom resequencing beads to 13 SCC-associated genes in the literature and resequenced each of the three cell groups to an



average coverage of 700× (22) (Fig. 3D). A variant was defined as a nucleotide change displaying a minimum alternative allele frequency of at least 5% and a minimum of 100 total reads for that locus. Applying these sensitive parameters, four to seven SCC-associated variants occurred with our manufacturing procedure, with two variants in *JAG2* and *TP53* present as germline variants in patients AO3 and AO1, respectively. Whereas some variants were present in the original donor cells (for example, Notch1 S1541R in AO3 KC), they were absent in the clonal iPSCs derived from them, providing additional evidence for the lack of selective mutational pressure. Moreover, one of the lines, F1-2, had fewer SCC-associated mutations than the other lines with only one germline variant (Fig. 3E). In contrast, another line (K3-4 CT147 L1) contained an additional mutation in *Notch2* (Notch 2_p.5_6del) that is nonsynonymous and predicted to be deleterious, and thus would be a less desirable choice. Targeted resequencing for SCC-associated genes allowed the choice of a corrected cell bank from which to manufacture downstream tissues.

Protocol for differentiation of iPSCs into homogeneous keratinocytes

Previous gene therapy protocols with retroviral-mediated somatic keratinocyte stem cells have generated keratinocyte epithelial sheets

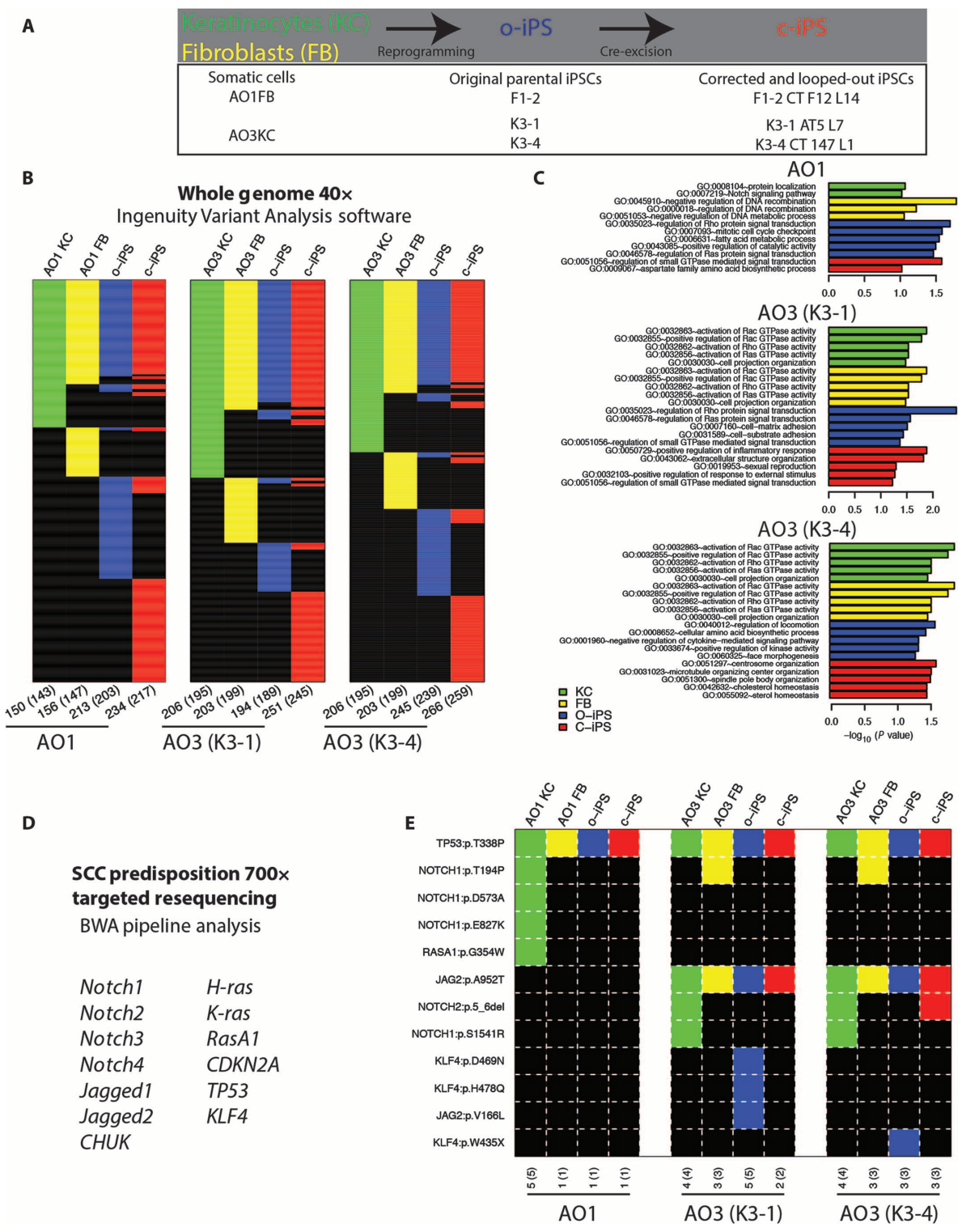


Fig. 3. Variant analysis from both whole-genome sequencing and targeted resequencing. (A) Experimental design to compare the sequence of two sets of patient somatic cells, original iPSCs (o-iPS), and corrected and looped-out iPSCs (c-iPS). (B) Heatmap of variants across keratinocytes (KC, green), fibroblasts (FB, yellow), original iPSC clones (o-iPS, blue), and corrected and Cre-excised iPSC clones (c-iPS, red) from whole-genome sequencing data. Each row represents a variant. Total number of variants for each sample

is given at the bottom (with number of genes having variants in parentheses). Black indicates the variants not observed in the sample. (C) Enriched GO terms for each set of variants from whole-genome sequencing. (D) Targeted SCC-predisposing genes for resequencing. (E) Twelve new functional variants across KCs, FBs, o-iPSCs, and c-iPSCs from targeted resequencing data. Total number of variants for each sample is given at the bottom (with number of genes having variants in parentheses). Black indicates the variants not observed.

for grafting with success in patients (9, 22). Having confirmed that several patient-derived iPSC lines meet our safety criteria for risk of developing SCC, we next developed a protocol to differentiate these cells into relatively pure cultures of functional keratinocytes that could be grown into epithelial sheets to restore adhesion in the skin (Fig. 4A). Previous studies indicated the ability to generate ES cell- and iPSC-derived keratinocytes, using a combination of retinoic acid (RA) and bone morphogenetic protein 4 (BMP4) (23–25) with varying efficiencies and cell heterogeneity. Here, we optimized and extended those prior findings to develop an improved protocol for generating epithelial sheets.

Prior studies are conflicting regarding the necessity for predifferentiation culture of iPSCs with feeders and the formation of embryoid bodies for optimal differentiation (26). To address this controversy, we tested the role of feeders by growing cells in mTeSR or feeder cell-

conditioned medium during maintenance phase and noticed a marked decrease in differentiation efficiency without conditioned medium (fig. S5A). Passaging cells onto feeders for five passages ameliorated the efficiency of differentiation, demonstrating that the effect is reversible. Previous studies in other tissues suggest that the formation of small, uniform embryoid bodies before the induction of differentiation generates more reproducible cultures (27, 28). We tested this hypothesis by plating the iPSCs in AggreWell 400 plates, with each well containing 1200 low-binding microwells within it (29). AggreWell-plated iPSCs resulted in more synchronous and larger keratinocyte colonies and were associated with decreased fibroblast and undifferentiated iPSC contamination (Fig. 4B and fig. S5B). We conclude that embryoid body formation and iPSC growth on feeders before differentiation improves efficiency and reduces heterogeneity of the keratinocytes culture.

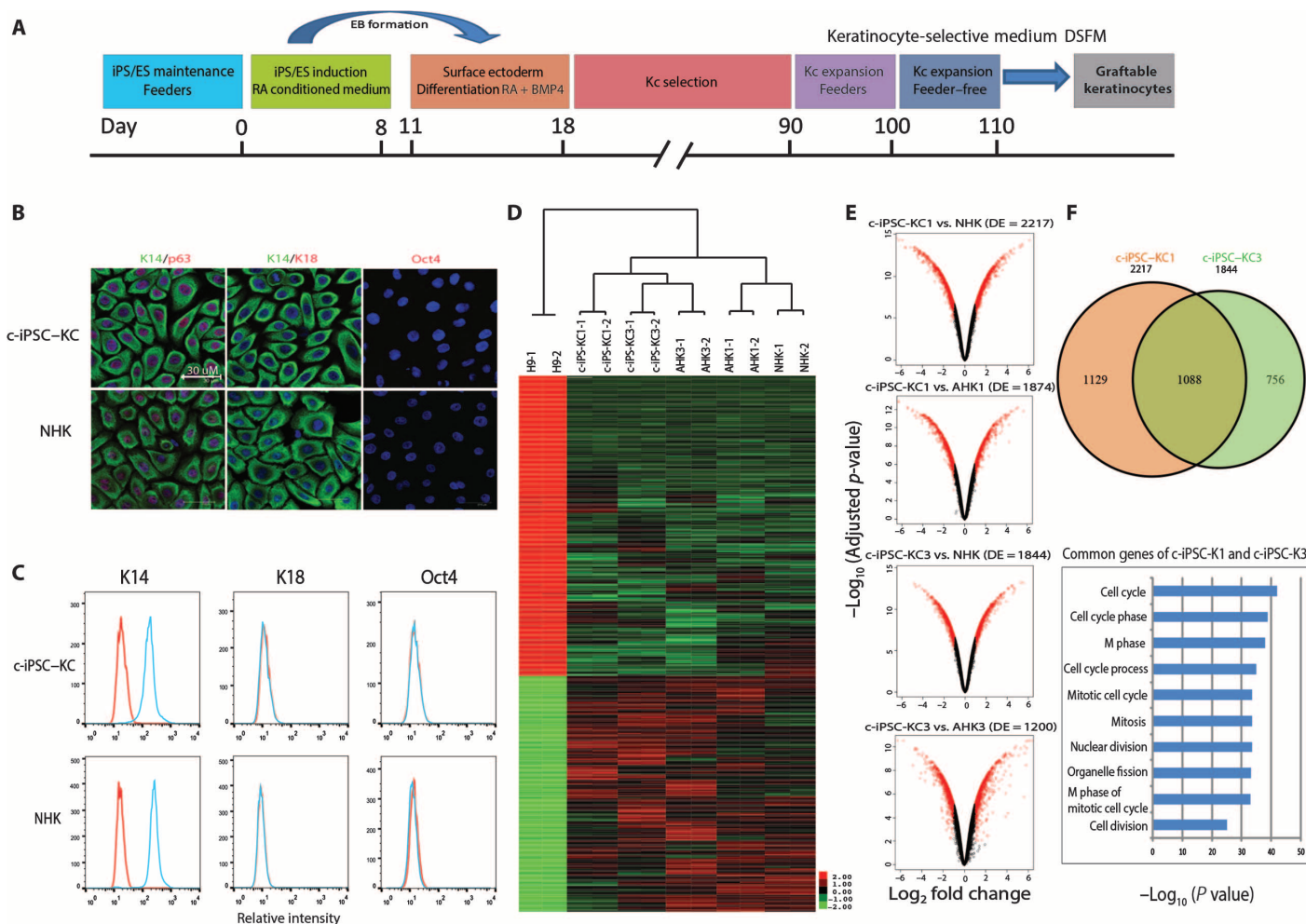


Fig. 4. Generation of pure, functional keratinocytes from patient-specific iPSCs. (A) Schematic diagram of iPSC differentiation to keratinocytes. (B) Immunofluorescence of K14, K18, p63, and Oct4 in iPS-KCs derived from patient-specific iPSCs (day 60) and in NHK. (C) Representative FACS analysis of K14, K18, and Oct4 in iPS-KCs derived from patient-specific iPSCs compared with NHKs. (D) Microarray analysis of two patient-specific iPSC-derived keratinocytes (iPS-KC1 and iPS-KC3), corresponding to patient keratinocytes AHK1 and AHK3, respectively. These cells were compared

with NHKs as well as hES cells (H9). All samples were analyzed in duplicate, and differential gene expression was measured as \log_2 fold change relative to H9. (E) Volcano plots comparing corrected iPS-KC lines, NHK, and AHK1. Differentially expressed (DE) genes are based on adjusted $P \leq 0.01$ [analysis of variance (ANOVA)] and fold change ≥ 2 (shown in red). (F) Venn diagram of DE genes from iPS-KC1 versus NHK (DE = 2217) and from iPS-KC3 versus NHK (DE = 1844). Enriched GO terms of the common DE genes (DE = 1088).

Using this improved protocol, we found that within 7 days after starting treatment with RA and BMP4, the culture began to mimic epidermal development and show reduced expression of pluripotency genes such as *Oct4* and up-regulation of epidermal genes like *p63*, *Keratin 18* (K18), and *Keratin 14* (K14). Over the course of 60 days, cells cultured in either N2 medium or defined keratinocyte serum-free medium (D-KSFM) transitioned through a $K14^+K18^+$ double-positive simple epithelial stage before maturing into a uniform population of $K14^+K18^-$ stratified epithelial cells (Fig. 4B and fig. S5C). Keratinocytes derived from genetically corrected iPSCs (c-iPS-KCs) were similar in morphology to neonatal human keratinocytes (NHKs), expressed p63 and K14 (markers of basal epithelia), and did not express K18 and OCT4 (markers of simple epithelia and pluripotency, respectively) (Fig. 4, B and C). To demonstrate the homogeneity of the final culture, we performed fluorescence-activated cell sorting (FACS) analysis of iPS-KCs for K14 and K18 to evaluate the purity of the cells and observed one $K14^+$ peak similar in purity and intensity to NHKs, indicating that the population of cells is homogeneous and is composed of fully mature $K14^+/K18^-$ keratinocytes (Fig. 4C).

We used our protocol to differentiate three o-iPSC lines (F1-2, K3-1, and K3-4) and three c-iPSC lines (F1-2 CTF12 L14, K3-1 AT5 L7, and K3-4 CT147 L1) and examined them for keratinocyte-like features. We were able to derive cells that closely resembled keratinocytes from all six lines (Fig. 4, D and E), demonstrating the reproducibility of our protocol. However, line-to-line variability was noted with respect to time to keratinocyte emergence, size of keratinocyte colony, and ability to form a stratified epidermis (fig. S5D).

To investigate how closely our c-iPS-KCs resembled keratinocytes from the same donor patient and NHKs, we performed global gene expression analysis of two corrected iPS-KC lines, one originating from fibroblasts (iPS-KC1) and one originating from keratinocytes (iPS-KC3), in addition to keratinocytes obtained from the same donor patients (AHK1 and AHK3), NHKs, and undifferentiated hES cells (H9) (Fig. 4D). The common up-regulated genes were associated with the GO terms “epidermal development,” “adhesion,” and “basement membrane formation,” indicating that iPS-KCs were turning on the keratinocyte program. There was also a large degree of overlap between iPS-KC, AHK, and NHK expression. Volcano plots of gene expression differences (significant expression differences are depicted in red in Fig. 4E) demonstrated that iPS-KCs were 93.6% (iPS-KC1) and 94.7% (iPS-KC3) similar to NHK, and 94.5% (iPS-KC1) and 94.1% (iPS-KC3) similar to their respective donor keratinocytes (AHK1 and AHK3) (Fig. 4, E and F).

To further analyze the differences between the iPS-KCs and normal keratinocytes, we identified genes differentially expressed in the iPS-KC lines relative to NHK and found that of the 2973 genes differentially expressed in either iPS-KC line, 1088 genes (37%) were common to both iPS-KC lines (Fig. 4F). These genes were significantly enriched for GO terms associated with “cell cycle” and “mitosis” compatible with the decreased proliferative capacity of iPSC-derived keratinocytes compared to primary keratinocytes (Fig. 4F and table S2). Together, these results suggest that our process of reprogramming and then differentiating donor cells generated cells that have properly differentiated and turned off pluripotency genes and are properly committed to the keratinocyte lineage, with consistent differences remaining in cell proliferation capacity.

Reconstitution of type VII collagen expression in corrected iPSC-derived keratinocytes

The similarity of gene expression between iPS-KCs and donor cells indicated that iPS-KCs have activated the keratinocyte program and

committed to the keratinocyte lineage. We investigated whether iPS-KCs could form stratified epidermis and ameliorate the RDEB phenotype by secreting collagen VII onto the basement membrane zone. Western blot confirmed expression of full-length wild-type collagen VII protein by the two corrected c-iPS-KC lines, but not by uncorrected o-iPS-KCs (note truncated band in o-iPS-KC3 that is corrected in c-iPS-KC3) (Fig. 5A).

To investigate whether this collagen VII was functional, we performed an in vitro skin reconstitution assay using rat-tail collagen dermis. A human-specific N-terminal antibody (LH7.2) indicated proper localization of human collagen VII to the basement membrane in corrected (c-iPS-KC1) but not in uncorrected (o-iPS-KC1) skin equivalents (Fig. 5, B and C). Staining with other basement membrane components, laminin 332 and integrin α_6 , and the cell-cell adhesion molecules E-cadherin and desmoglein 3 revealed a typical epidermal differentiation pattern (Fig. 5B). Furthermore, both o-iPS-KCs (Fig. 5C) and c-iPS-KCs (Fig. 5B) formed a stratified epidermis in vitro, with a K14-expressing basal layer and suprabasal layers expressing keratin 10 (K10) and human-specific involucrin, demonstrating that the two cell lines only differed by the ability to express human COL7A1.

We further investigated whether corrected iPS-KCs had the potential to maintain the skin long-term in vivo by performing xenografts onto immunocompromised mice, a validated preclinical model for human epithelial sheet formation (30). The grafted c-iPS-KC1 cells could rebuild fully stratified and mature skin in 3 weeks (Fig. 5D and fig. S6). Using both the collagen VII N-terminal LH7.2 and C-terminal LH24 antibodies, we showed that full-length and functional collagen VII was produced by the keratinocytes, secreted and deposited into the basement membrane zone. Like the organotypic cultures, staining with differentiation markers K14, K10, involucrin, laminin 332, integrin α_6 , E-cadherin, and desmoglein 3 demonstrated the differentiation into a stratified epidermis. Whereas iPS-KC-derived epidermis could survive 3 weeks after grafting, long-term grafts lasting more than 1 month thus far have been unsuccessful.

DISCUSSION

The use of human iPSCs for regenerative medicine is an attractive alternative for the treatment of degenerative diseases in which an extensive or continuous need of tissue regeneration is required. iPSCs can be expanded indefinitely retaining a pluripotent and undifferentiated state and can therefore be used as a constant source of material for cell therapy. Development of iPSC-based therapies for RDEB patients represents an ideal paradigm owing to the severe nature of the disease, the demonstration that corrected keratinocytes can have long-term tissue repopulation, and the need for large numbers of stem cells to cover the affected surface area. In addition, iPSCs are more easily amenable to in situ gene correction. Of note, two accompanying studies demonstrate the relevance of iPSCs for the clinical treatment of EB in a murine model (31) and in naturally occurring revertant keratinocytes from patients affected by junctional EB (17). Despite the great potential of iPSCs, a conspicuous body of evidence has shown that more accurate and stringent standards of characterization of iPSC clones need to be put in place for the development of reliable and practical clinical protocols that will not affect the safety of the patients. Our results provide a platform for the development of protocols using iPSCs for the treatment of RDEB and set a preliminary set of standards for the clinical application of iPSCs.

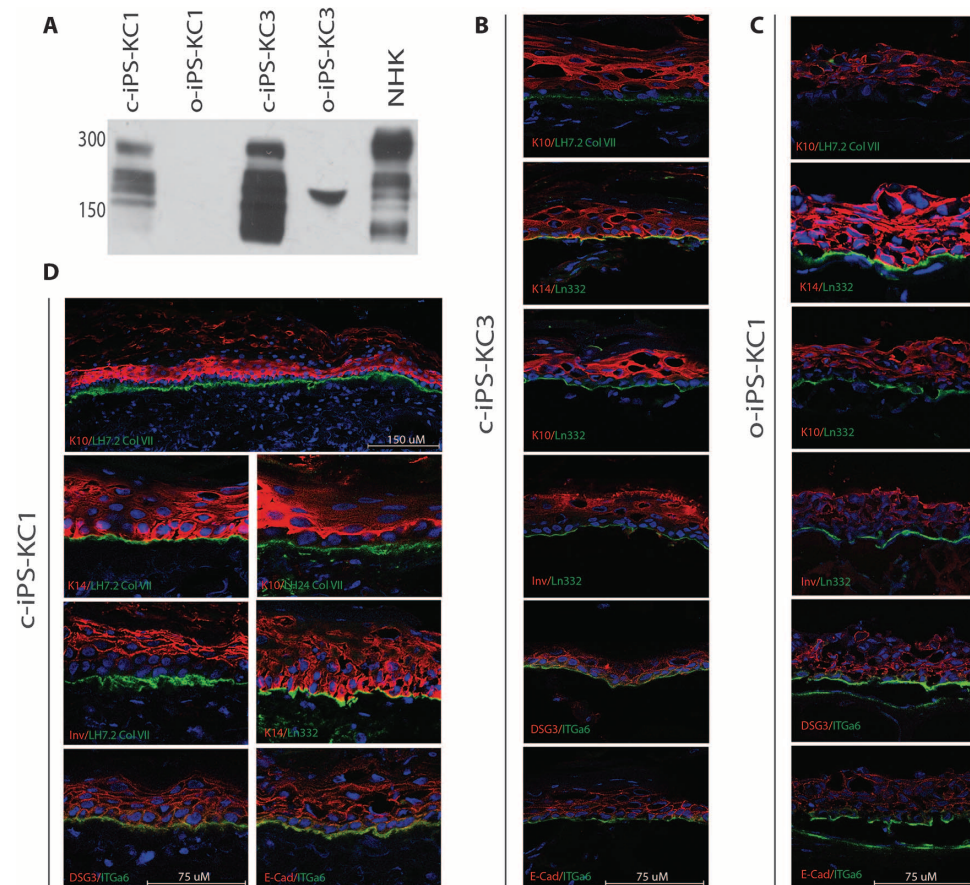


Fig. 5. Keratinocytes derived from patient-specific iPSCs express wild-type collagen VII and stratify in vitro and in vivo. (A) Full-length collagen VII (~300 kD) was expressed in mutation-corrected keratinocytes derived from patient-specific iPSCs (c-iPS-KC1 and c-iPS-KC3) and normal keratinocytes (NHK), but not in keratinocytes from uncorrected iPSC cells (o-iPS-KC1 and o-iPS-KC3). (B) Organotypic culture of corrected c-iPS-KCs on a rat type I collagen lattice. Immunofluorescence staining with antibodies to the following differentiation markers: human N-terminal collagen VII (LH7.2), K14, K10, involucrin (Inv), DSG3, E-cadherin (E-Cad), integrin α_6 (ITGa6), and laminin 332 (Ln332). (C) Organotypic culture of noncorrected o-iPS-KCs on rat type I collagen lattice with antibodies as in (B). (D) Three-week xenograft of c-iPS-KCs onto nonobese diabetic severe combined immunodeficient γ (NSG) mice. Top image: Low-power view revealing the corrected epidermis expressing human type VII collagen and K10. All other images show stratification of c-iPS-KC-derived epidermis, using the N-terminal (LH7.2) and C-terminal (LH24) type VII collagen antibodies and the indicated differentiation markers as in (B).

Although there are methods that, in principle, could generate a corrected, patient-specific iPSC bank, we show the utility of sequential excisable reprogramming factor lentiviral and genome editing AAV-DJ transductions. Previous studies have examined the ability to either reprogram somatic cells to iPSCs, or to undergo genome editing. With the goal of generating iPSCs and correcting their genetic defects, integrating reprogramming vectors and genome editing steps allows one to better control and track the genomic modifications once the desired modifications have been achieved. In contrast, nonintegrating reprogramming methods might still lead to random integrations, which are difficult to exclude. Remaining in the cell bank are two unique loxP recombination sites that can be easily identified and used to barcode and track the fate of cells in subsequent tissue engineering applications, facilitating pivotal pharmacology and toxicology studies and first-in-human trials. Because of the experience and relatively straightforward GMP production of

both lentiviral and adenoviral vectors, the sequential L4F-AAV-DJ approach can also be scaled to the industrial levels needed in many clinical applications.

Our studies place the recently identified highly recombinogenic AAV-DJ variant among the top approaches to be considered in the armamentarium of iPSC genome editing tools. Optimal genome editing requires cellular entry of editing enzymes, a site-specific double-strand DNA break, and a nucleic acid template for repair. We initially explored conventional targeting methods, given the potential safety concerns around engineered proteins with nuclease activity such as ZFNs, TALENs, and CRISPR/Cas9 enzymes. However, cell type-specific tropism accompanied by a high site-specific recombination frequency indicates that AAV-DJ encompasses all three editing requirements in one reagent. In contrast to conventional targeting, AAV-DJ packaging limits the recombination ability to a smaller region, encompassing six exons in our study. However, several AAV-DJ targeting vectors could be created to allow targeting in the majority of the *COL7A1* locus.

Although a corrected iPSC bank could be used to treat a variety of tissues dependent on type VII collagen for function, including bone marrow or esophagus, our study improves the iPSC differentiation over previous protocols (23–27) and allows the generation of keratinocytes from multiple independent pluripotent stem cell lines including RDEB patient-derived and genetically corrected lines. One critical detail for this optimization was the formation of microscopic embryoid bodies of uniform size and shape before differentiation, which promotes a more uniform

response to differentiation conditions. Although in our study AggreWell 400 plates gave optimal results for keratinocyte generation using BMP and RA, other three-dimensional orientation and cell densities may be required in other tissue generation protocols. Consistent with the relatively synchronous cultures, FACS analysis shows a uniform population of mature keratinocytes lacking the immature marker K18. Of importance was the absence of undifferentiated pluripotent cells in the mature cultures (level of detectability: 1 in 100,000), suggesting that the risk of potential teratoma formation after transplantation is extremely low. Moreover, it is unclear whether undifferentiated iPSCs lacking appropriate adhesion molecules could incorporate into a stratified epithelial tissue if present.

In vitro and in vivo epidermal regeneration assays demonstrated that these keratinocyte cultures stratify and deposit type VII collagen, confirming the ability to differentiate the iPSCs into epidermal sheets.

Because of our group's previous phase 1 clinical trial demonstrating the ability of retrovirally infected somatic keratinocytes to correct blistering defects in RDEB patients (9), our data provide strong support for the clinical utility of our manufacturing protocol. Keratinocytes generated through our protocol could be used in similar epithelial sheets for treating nonhealing patient wounds.

Although the present animal studies demonstrated the ability of the keratinocytes to stratify and incorporate type VII collagen, to date we have not been able to generate iPSC-KCs with long-term graftability on mouse skin. We have excluded epigenetic memory because both fibroblast- and keratinocyte-derived iPSCs demonstrate similar restrictions. By contrast, the comparison between iPSC-KCs and NHKs or donor keratinocytes revealed alterations in the expression of cell cycle and mitotic genes (Fig. 4, E and F). These expression differences suggest an increased senescence that limits the long-term tissue contribution, although human epithelium may function with improved survival when grafted onto patients rather than as a xenograft. Our group has successfully grafted corrected epithelial sheets in patients affected by RDEB (9); however, keratinocyte grafting of burn wounds will require composite grafts that provide additional mechanical strength and long-term stability (32). Future studies should focus on improving efficiency and decreasing line-to-line variability of the differentiation protocol to remove dependency on murine feeders and to prospectively identify cell surface molecules associated with long-term progenitors.

Our results demonstrate the critical need and distinct advantage for extensive genetic analysis of the corrected iPSC bank before release for tissue production. This has become an emerging problem because recent works have shown that methods of derivation and prolonged culture might lead to the accumulation of mutations that could have unpredictable effects on the patients upon transplantation. Our analysis suggests a lack of distinct mutational selection during reprogramming and correction, although we cannot rule out the random occurrence of a deleterious mutation appearing. However, in the case of comorbidities, such as SCC formation in RDEB patients, the ability to sequence a stable cell bank allows one to produce a corrected and genetically "clean" source for tissue engineering. In theory, this approach could be used to qualify cell banks for any tissue derived from a donor tissue that has accumulated somatic variants, using any targeted genes of interest.

MATERIALS AND METHODS

Study design

The current study aimed to develop a clinical platform for the treatment of RDEB using autologous iPSCs. We performed iPSC derivation, genomic in situ correction, and whole-genome sequence analysis on several iPSC clones derived from distinct somatic cell types from 18 patients to assess the feasibility and reproducibility of our results. Specific assays were performed in replicates to determine statistical significance. Blinding was applied to determine HR events, pluripotency of iPSCs, whole-genome sequencing, and targeted resequencing.

Patient selection

RDEB patients ($n = 18$) were enrolled in clinical studies approved by the Stanford Institutional Review Board (IRB) (Study Protocols 8557, 15898, and 17158, with Clinicaltrials.gov identifiers NCT00533572, NCT00904163, and NCT01019148, respectively). Declaration of Helsinki protocols were followed, and all subjects gave written informed consent before performing

any study procedures. Those that passed additional selection criteria (age, presence/absence of immunogenic NC1, clinical status compatible with the development of the study) were asked to participate in the study and donate cells.

Cell line nomenclature

The three patients from whom we obtained skin biopsies were called AO1, AO2, and AO3. Fibroblasts (FB) and keratinocytes (KC) isolated from the patients were named AO1 FB, AO1 KC, AO2 FB, AO2 KC, AO3 FB, and AO3 KC. The original untargeted iPSC lines (o-iPSCs) derived from patient AO1 were obtained from fibroblasts and were called F1-1, F1-2, and F1-3. The original iPSC lines derived from patient AO2 were obtained from fibroblasts and were called F2-3 and F2-13. The original iPSC lines derived from patient AO3 were obtained from both fibroblasts (F3-1, F3-2, and F3-3) and keratinocytes (K3-1 and K3-4). Targeting of the *COL7A1* locus and successful excision of both the reprogramming cassette and the positive selection cassette were achieved in patient AO1- and patient AO3-derived iPSCs, using conventional targeting and AAV-mediated targeting (c-iPSCs). The clones that were karyotypically normal were used in the study and were named as follows: F1-2 CTF12 L14 (conventional targeting), F1-1L8 AT5 L7 (AAV-mediated targeting), K3-4 CT147 L1 (conventional targeting), and K3-1 AT5 L5 (AAV-mediated targeting).

Cell-reprogramming lentivirus production

Plasmids for the production of the polycistronic lentiviral reprogramming vector were donated by G. Mostoslavsky (Boston University School of Medicine). Lentivirus production was performed as described previously (33). Viral supernatant was harvested five times every 12 hours beginning at 48 hours after transfection, and ultracentrifugation was used to concentrate the virus. Viral particles were resuspended in a volume of phosphate-buffered saline 1/100th that of the original supernatant. Aliquots of concentrated virus were stored at -80°C .

GMP lentiviral vector manufacturing

Lentiviral vector manufacturing was carried out in the University of California Davis GMP facility applying standard operating procedures and quality control, as described in Supplementary Materials and Methods.

Derivation and culture of patient iPSCs

iPSCs were derived under GMP conditions, as described in Supplementary Materials and Methods. Dermal fibroblasts and keratinocytes from individuals with RDEB were derived and cultured from a $4 \times 4\text{-mm}^2$ skin biopsy in agreement with the Stanford IRB protocol. The skin biopsy was aseptically minced into small pieces and cultured in Dulbecco's modified Eagle's medium + 10% fetal bovine serum (FBS) [mouse embryonic fibroblast (MEF) medium] under sterile glass coverslips to promote adherence to the plastic dishes in gelatinized 60-mm tissue culture plates. Fibroblasts and keratinocytes were infected with the polycistronic stem cell cassette (STEMCCA) (33) lentiviral reprogramming vector. Cells (10^5) were seeded in corresponding culture medium and infected 24 hours later by overnight incubation with STEMCCA lentivirus with polybrene (8 $\mu\text{g}/\text{ml}$). Cells were then washed, kept in culture medium for 6 days, and transferred onto inactivated MEFs. The following day, medium was replaced with hES medium, and the cells were grown for up to 8 weeks until hES-like colonies started to emerge. iPSC colonies were manually picked and expanded on MEFs. After about 10 passages,

the clones were transferred to feeder-free culture conditions using mTeSR1 (STEMCELL Technologies) according to the manufacturer's instructions.

Conventional and AAV-mediated gene targeting

Targeting methods are described in Supplementary Materials and Methods.

Differentiation of iPSCs into keratinocytes (iPS-KCs)

We devised a protocol for differentiating patient-derived iPSCs into keratinocytes by modifying protocols reported for H9 hES cells (22–24). iPSCs were maintained on mitomycin-treated CF1 MEFs in w8 medium (<http://www.wicell.org>) supplemented with recombinant human fibroblast growth factor-2 (8 ng/ml; PeproTech). Before differentiation, iPSCs were passaged onto CELLstart (Life Technologies, a xeno-free and defined coating matrix)-coated dishes in medium conditioned with MEFs for two passages. For the last 2 days, the medium was supplemented with RA (1 µg/ml; Sigma-Aldrich).

To induce differentiation into keratinocytes, iPSCs were first formed into embryoid bodies, using AggreWell 400 plates and AggreWell medium (STEMCELL Technologies) supplemented with RA and 10 µM ROCK inhibitor (STEMCELL Technologies) for 24 hours. Embryoid bodies were collected and cultured in suspension for 2 days and then plated onto gelatin-coated dishes in FAD medium (24) for 4 days and then in N2 medium (34) for 3 days. During this 7-day differentiation, the media were supplemented with RA (1 µg/ml) and human recombinant BMP4 (25 ng/ml; R&D Systems). The medium was then changed to either N2 or D-KSFM (Life Technologies), and the cells underwent selection and expansion for 2 months. The resulting colonies were passaged onto mitomycin-treated MEFs for one passage, and then onto CELLstart-coated plates before being analyzed by immunostaining and functional studies.

In vitro skin reconstitution assay

We performed in vitro skin reconstitution assays on fibroblast-populated collagen lattices, as described previously (35). Briefly, 750,000 mouse neonatal fibroblasts were mixed with a solution of rat-tail type I collagen (BD Biosciences). After 4 days, it formed a lattice, which was used as a dermal equivalent. iPS-KCs (7.5×10^5) were seeded on top of the lattice and cultured submerged for 5 days. The medium was then changed to Keratinocyte Growth Medium for 5 days, after which stratification was induced by raising the collagen lattice to air-liquid interface. After 2 weeks, the collagen lattices were collected, fixed in 4% paraformaldehyde, and embedded in optimum cutting temperature compound (OCT) and paraffin for immunofluorescence analysis.

Mouse skin xenografts

All animal experiments followed the NIH (National Institutes of Health) *Guide for the Care and Use of Laboratory Animals* under Stanford APLAC (Administrative Panel on Laboratory Animal Care) protocol #11680. Xenograft protocol was performed as described previously (35). iPS-KCs (7.5×10^5) were seeded onto a 1.5-cm² piece of devitalized human dermis (New York Firefighter Skin Bank) and grown in D-KSFM for 10 days, followed by Keratinocyte Growth Medium for 5 days. Next, the pieces were grafted onto the backs of NSG mice for 2 to 4 weeks. Upon collection, the pieces were embedded in OCT and paraffin for immunofluorescence analysis (Supplementary Materials and Methods).

Statistical analysis

Microarray data normalization was performed by quantile normalization and log₂ transformation. Differentially expressed genes were identified on the basis of adjusted $P \leq 0.01$ (ANOVA) and fold change ≥ 2 . Data analysis on whole-genome sequencing was performed by using IVA tool. Functional profiling using GO enrichment was performed by DAVID.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Immunohistochemical characterization of patient-specific iPS clones.

Fig. S2. GMP production of patient-specific iPS clones.

Fig. S3. Genetic and karyotypic characterization of patient-specific iPSC clones after loop-out.

Fig. S4. CRISPR versus AAV-DJ targeting efficiency at the *LAMA3* locus in *LAMA3*-deficient primary keratinocytes.

Fig. S5. Optimization and validation of keratinocyte differentiation protocol.

Fig. S6. Histology of corrected iPSC xenograft.

Table S1. Persistent variant genes during corrected iPSC cell generation.

Table S2. List of genes, categorized by GO term, differentially expressed between iPS-KC and NHK. References (36–39)

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Acknowledgments: We thank P. Khavari and C. Lee for sequencing reagents before publication and for help with the targeted resequencing, and I. Caras and E. Feigal for project advice. **Funding:** The California Institute for Regenerative Medicine (DR1-01454), Children's Health Research Institute (S.P.M.), Howard Hughes Medical Institute (T.L.L. and S.K.K.), and NIH 5R01 ARO55914 (Z.S., A.T., and A.T.L.). M.W. is a New York Stem Cell Foundation-Robertson Investigator and a Tashia and John Morgridge Faculty Scholar, Child Health Research Institute at Stanford. **Author contributions:** Developed concept and project management: A.E.O., M.W., and A.T.L.; developed recombination protocols: P.W., T.L.L., S.K.K., V.S., L. Lisowski, M.A.K., E.B., S.P.M., A.E.O., and M.W.; developed keratinocyte protocol: H.H.Z., E.B., S.P.M., M.A., S.L., L. Li, and A.E.O.; developed iPSC protocol: V.S., B.H.D., J.H., and M.W.; GMP vector studies: A.S., G.B., V.S., B.H.D., and M.W.; patient recruitment and regulatory: Z.S., A.T., and A.T.L.; cell bank sequencing: J.L., V.S., M.W., and A.E.O. All authors contributed to the writing of the manuscript. **Competing interests:** M.K. has one patent on AAV-DJ. **Data and materials availability:** Plasmids for AAV-DJ need a material transfer agreement from Stanford University.

Submitted 15 May 2014

Accepted 7 November 2014

Published 26 November 2014

10.1126/scitranslmed.3009540

Citation: V. Sebastiano, H. H. Zhen, B. H. Derafshi, E. Bashkurova, S. P. Melo, P. Wang, T. L. Leung, Z. Siprashvili, A. Tichy, J. Li, M. Ameen, J. Hawkins, S. Lee, L. Li, A. Schwertschkow, G. Bauer, L. Lisowski, M. A. Kay, S. K. Kim, A. T. Lane, M. Wernig, A. E. Oro, Human *COL7A1*-corrected induced pluripotent stem cells for the treatment of recessive dystrophic epidermolysis bullosa. *Sci. Transl. Med.* **6**, 264ra163 (2014).