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Induced Pluripotent Stem Cells from Individuals with Recessive Dystrophic Epidermolysis Bullosa

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Recessive dystrophic epidermolysis bullosa (RDEB) is an inherited blistering skin disorder caused by mutations in the *COL7A1* gene-encoding type VII collagen (Col7), the major component of anchoring fibrils at the dermal–epidermal junction. Individuals with RDEB develop painful blisters and mucosal erosions, and currently, there are no effective forms of therapy. Nevertheless, some advances in patient therapy are being made, and cell-based therapies with mesenchymal and hematopoietic cells have shown promise in early clinical trials. To establish a foundation for personalized, gene-corrected, patient-specific cell transfer, we generated induced pluripotent stem (iPS) cells from three subjects with RDEB (RDEB iPS cells). We found that Col7 was not required for stem cell renewal and that RDEB iPS cells could be differentiated into both hematopoietic and nonhematopoietic lineages. The specific epigenetic profile associated with de-differentiation of RDEB fibroblasts and keratinocytes into RDEB iPS cells was similar to that observed in wild-type (WT) iPS cells. Importantly, human WT and RDEB iPS cells differentiated *in vivo* into structures resembling the skin. Gene-corrected RDEB iPS cells expressed Col7. These data identify the potential of RDEB iPS cells to generate autologous hematopoietic grafts and skin cells with the inherent capacity to treat skin and mucosal erosions that typify this genodermatosis.

Journal of Investigative Dermatology (2011) **131,** 848–856; doi:10.1038/jid.2010.346; published online 2 December 2010

INTRODUCTION

The severe blistering disease recessive dystrophic epidermolysis bullosa (RDEB) is caused by loss-of-function mutations in the type VII collagen gene, *COL7A1* (Shimizu *et al.*, 1996; Varki *et al.*, 2007; Dang and Murrell, 2008). Type VII collagen (Col7) deficiency is responsible for diminished or absent formation of homotrimers of Col7, necessary to form anchoring fibrils that normally insert into the lamina densa of the skin basement membrane and to provide adhesion to interstitial dermal collagen. Without anchoring fibrils, skin integrity is lost and blistering occurs below the lamina densa. Individuals with RDEB exhibit extensive painful trauma-induced skin and mucosal blisters and erosions. Owing to extreme skin fragility, recurrent injury, and aberrant tissue repair, subjects with severe generalized RDEB are prone to develop esophageal strictures, mutilating scarring, pseudosyndactyly, joint contractures, and highly malignant skin squamous cell carcinomas (Fine *et al.*, 2008). Until recently, treatment options have been limited to palliative measures, such as analgesia, bandaging, nutritional support, esophageal dilatation, and treatment of infections (Mellerio *et al.*, 2007; Abercrombie *et al.*, 2008). At present, however, several promising interventions are being developed, including systemic interventions using gene therapy, protein therapy, and cellular transplantation (Chen *et al.*, 2002; Mecklenbeck *et al.*, 2002; Ortiz-Urda *et al.*, 2002, 2003; Fivenson *et al.*, 2003; Woodley *et al.*, 2004a, b, 2007; Ferrari *et al.*, 2006; Chino *et al.*, 2008; Wong *et al.*, 2008; De Luca *et al.*, 2009; Tolar *et al.*, 2009; Conget *et al.*, 2010; Yan and Murrell, 2010). Cellular transplantation studies using mesenchymal stem cells or hematopoietic stem and progenitor cells have been shown to provide a source of normal donor Col7 and to ameliorate many of the disease manifestations (Chino *et al.*, 2008; Tolar *et al.*, 2009; Conget *et al.*, 2010). However, these therapies have focused on using allogeneic cells, which may be targeted for elimination by the host immune system. In addition, for allogeneic hematopoietic cell transplantation, potentially toxic chemoradiotherapy and immunesuppressive agents need to be used to prepare the host to receive the allogeneic donor graft.

Patient-specific stem cells, such as the recently identified induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006a; Yu *et al.*, 2007; Park *et al.*, 2008b), present an opportunity to use the progeny of autologous

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Abbreviations: Col7, type VII collagen; DAPI, 4,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; FB, fibroblast; iPS, induced pluripotent stem; KC, keratinocyte; MEF, murine embryonal fibroblast PBS, phosphate-buffered saline; RDEB, recessive dystrophic epidermolysis bullosa; WT, wild type

Received 15 June 2010; revised 27 September 2010; accepted 10 October 2010; published online 2 December 2010

rather than allogeneic cells to correct RDEB. An advantage of gene-corrected autologous RDEB iPS cells is the possibility of simultaneously deriving Col7-producing ectodermal tissues (such as the skin) and mesodermal tissues (such as hematopoietic stem and progenitor cells). As the systemic use of bone marrow and cord blood transplantation combined with local injections of skin fibroblasts (FBs) may be synergistic in the wound treatment of RDEB patients, autologous gene-corrected iPS cells would represent a robust approach to RDEB treatment by providing a virtually limitless supply of both cell types, while avoiding the need for potent immunesuppressive therapy to prevent an allogeneic immune response. Induction of iPS cells from RDEB subjects may also provide a means of better understanding the sequence of downstream events initiated by Col7 deficiency during the development of epithelial stem and progenitor cells, as well as their progeny, as assayed both *in vitro* and *in vivo*. Such knowledge may lead to innovative treatment approaches that could benefit individuals with RDEB, other related inherited skin diseases, and perhaps additional extracellular matrix disorders.

In this study, we show that iPS cells can be obtained from both skin FBs and keratinocytes (KCs) of individuals with RDEB (RDEB iPS cells). We report that RDEB iPS cells can be differentiated into both hematopoietic and nonhematopoietic cells. These data underscore the potential of RDEB iPS cells in modeling RDEB in the development and future use of gene-corrected RDEB iPS cells to generate autologous hematopoietic grafts for systemic therapy, as well as generating skin cells with the potential to treat individual skin and mucosal lesions that underscore RDEB.

RESULTS

RDEB iPS cells can be generated from both FBs and KCs

Col7 can be secreted by both FBs and KCs. Therefore, as both cell phenotypes are likely to be relevant to RDEB pathogenesis, we isolated FBs and KCs from the skin of three individuals with RDEB (Table 1). The cultured primary FBs and KCs had the characteristic spindle-shaped and cobblestone appearances, respectively and, as expected, KCs expressed Col7, type XVII collagen, keratin 1 (K1), keratin 5 (K5), and keratin 15 (K15) (Supplementary Figure S1 online).

To derive RDEB iPS cells, RDEB FBs (from patient 1 (P1) and patient 3 (P3)) and RDEB KCs (from patient 2 (P2) and P3) (Table 1) were transduced with retroviral vectors carrying four known reprogramming transcription factors (namely *OCT4*, *SOX2*, *KLF4*, and *c-MYC*), which are typically associated with pluripotency. Transduced cells were cultured on a supportive stroma of irradiated murine embryonal fibroblasts (MEFs). After 6–7 weeks, flat colonies of RDEB iPS cells with clear-cut, round edges emerged from the two-dimensional MEF culture (Figure 1a–o). Although both FBs and KCs have been used successfully to generate iPS cells, KCs have been preferred by some because they appeared to reprogram faster than FBs (Maherali *et al.*, 2008a). All iPS colonies in this study, however, appeared 45 ± 4 days after viral transduction, and with roughly similar efficiency in FB and KC cultures (Table 1).

When compared with parental RDEB KCs, RDEB iPS cells showed persistent mRNA expression of *SOX2*, used for reprogramming, as well as genes associated with pluripotency in iPS cells (such as *NANOG*, *LIN28*, and *DNMT3b*). Moreover, there was transient mRNA expression of *OCT4*, *c-MYC*, and *KLF4*, used to accomplish reprogramming. The mRNA profile seen in RDEB iPS cells is as would be expected to occur in wild-type (WT) iPS (WT iPS) cells (Figure 1p and q). RDEB iPS cells also expressed protein markers characteristic of reprogrammed iPS and embryonic stem cells: TRA-1-81, stage-specific embryonic antigens-3 and -4, OCT4, and NANOG (Figure 2 and Supplementary Figures S2 and S9 online).

These reprogramming factors are believed to activate a network of transcriptional factors, which in turn induce epigenetic changes (Freberg *et al.*, 2007; Aasen *et al.*, 2008; Okita *et al.*, 2008; Maherali *et al.*, 2008a; Park *et al.*, 2008a). Therefore, we used bisulfite sequencing to confirm the methylation status of *OCT4* and *NANOG* promoters in RDEB iPS cells. A methylated pattern is indicative of gene silencing, whereas an unmethylated pattern indicates the potential for robust gene expression. Individual colonies of cells from RDEB iPS cells were analyzed by bisulfite sequencing that showed a pattern characteristic of iPS cells in which OCT4 and NANOG promoter sequences are unmethylated. In contrast, mature progeny such as FBs and KCs had the expected pattern of both methylated and unmethylated

Table 1. Patient and iPS cell characteristics

Patient	Type VII collagen mutations	Age (years)	Cell type	Number of iPS colonies per 100,000 cells
P1	c.6176A>G (p.Glu2059Gly) IVS5+1G>A	6.2	FB	3
P2	c.4919delG (p.Gly1640fsX70) c.8254_8255delAG (p.Arg2751fsX20)	6.9	KC	7
P3	c.6781C>T (p.Arg2261X) IVS110-1 G>C	14.5	FB	2
P3	c.6781C>T (p.Arg2261X) IVS110-1 G>C	14.5	KC	2
WT	NA	9	KC	2

Abbreviations: FB, fibroblast; iPS, induced pluripotent stem; KC, keratinocyte; NA, not applicable; P, patient.

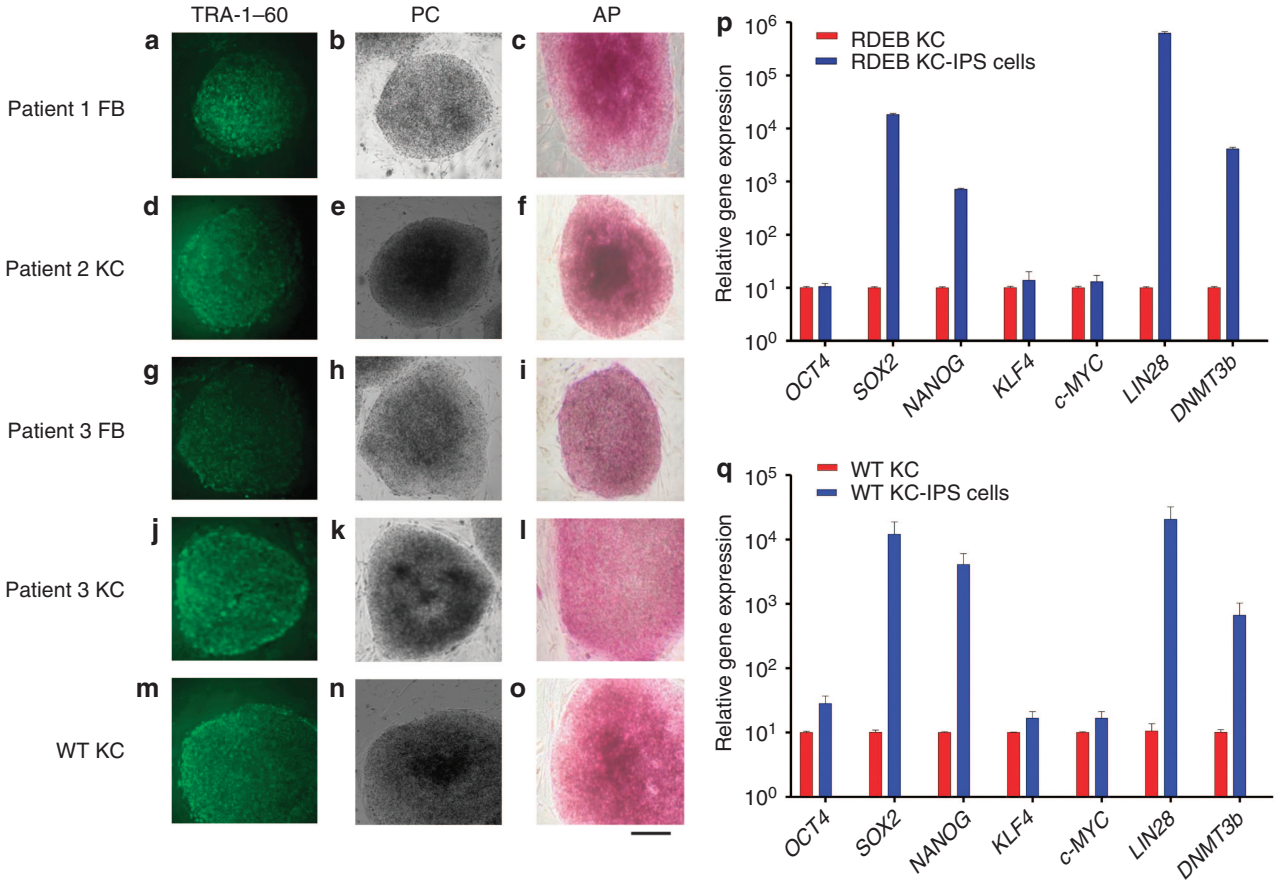

Figure 1. Induction of RDEB skin cells into RDEB iPS cells. (**a**, **d**, **g**, **j**, **m**) Live human iPS cultures stained with TRA-1-60 antibody 4 weeks after transduction. (**b**, **e**, **h**, **k**, **n**) Phase-contrast image of the same iPS colonies. (**c**, **f**, **i**, **l**, **o**) Alkaline phophatase stain. (**p**) Quantitative RT-PCR analysis of *OCT4*, *SOX2*, *NANOG*, *KLF4*, *c-MYC*, *LIN28*, and *DNMT3b* expression levels in RDEB KC iPS cells from P2 and P3 relative to expression levels in parental RDEB KCs from P2 and P3. (**q**) Quantitative RT-PCR analysis of the same genes in WT KC iPS cells relative to expression levels in parental WT KCs. All values were normalized against endogenous *GAPDH* expression. AP, alkaline phosphatase; FB, fibroblast; iPS cells, induced pluripotent stem cells; KC, keratinocyte; PC, phase contrast; P2, patient 2; P3, patient 3; RDEB, recessive dystrophic epidermolysis bullosa; RT-PCR, reverse transcriptase-PCR; WT, wild type. Bar = 50 μm.

sequences (Park *et al.*, 2008b; Raya *et al.*, 2009) (Figure 3 and Supplementary Figure S3 online).

Both RDEB FB- and RDEB KC-derived RDEB iPS cells from the three individuals, as well as the one WT control, had normal female karyotypes as determined by high-resolution chromosomal G-banding (Supplementary Figure S4 online and data not shown). As the purity of iPS cultures is critical for our downstream experimentation, we formally demonstrated a lack of contamination of stromal cell (MEF) feeder cells used to generate RDEB iPS cells. Competitive PCR of variable number tandem repeats indicated that all RDEB iPS cell lines were fully host derived (data not shown). Collectively, the transcription profile and cellular phenotype of RDEB iPS cells from all subjects were consistent with morphological and phenotypical gain of pluripotency.

RDEB iPS cells widely differentiate *in vivo*

To provide further evidence of the identity of iPS cells on a functional level, KC-derived RDEB iPS cells were injected intramuscularly into immunedeficient mice. Within 6–8 weeks, well-differentiated cystic teratomas were observed, confirming the phenotype-defining ability of iPS cells to differentiate *in vivo* into a wide array of cell lineages encompassing cells of endodermal, mesodermal, and ectodermal origins (Figure 4).

RDEB iPS cells differentiate into hematopoietic cells

As we have shown that cells contained in the bone marrow and cord blood can home to RDEB skin and produce Col7, one of the mesodermal lineages, hematopoietic lineage, is directly relevant to our ultimate goal of generating gene-corrected cells for autologous hematopoietic cell transplantation. The hematopoietic potential of RDEB iPS cells was tested using embryoid bodies (Supplementary Figure S5A online). Embryoid bodies mimic early stages of embryonic development and serve to provide three-dimensional architecture, which promotes cell differentiation. Embryoid bodies derived from RDEB iPS cells were enzymatically dissociated into single cells. After seeding in low-attachment dishes, cells were induced to differentiate in medium containing human

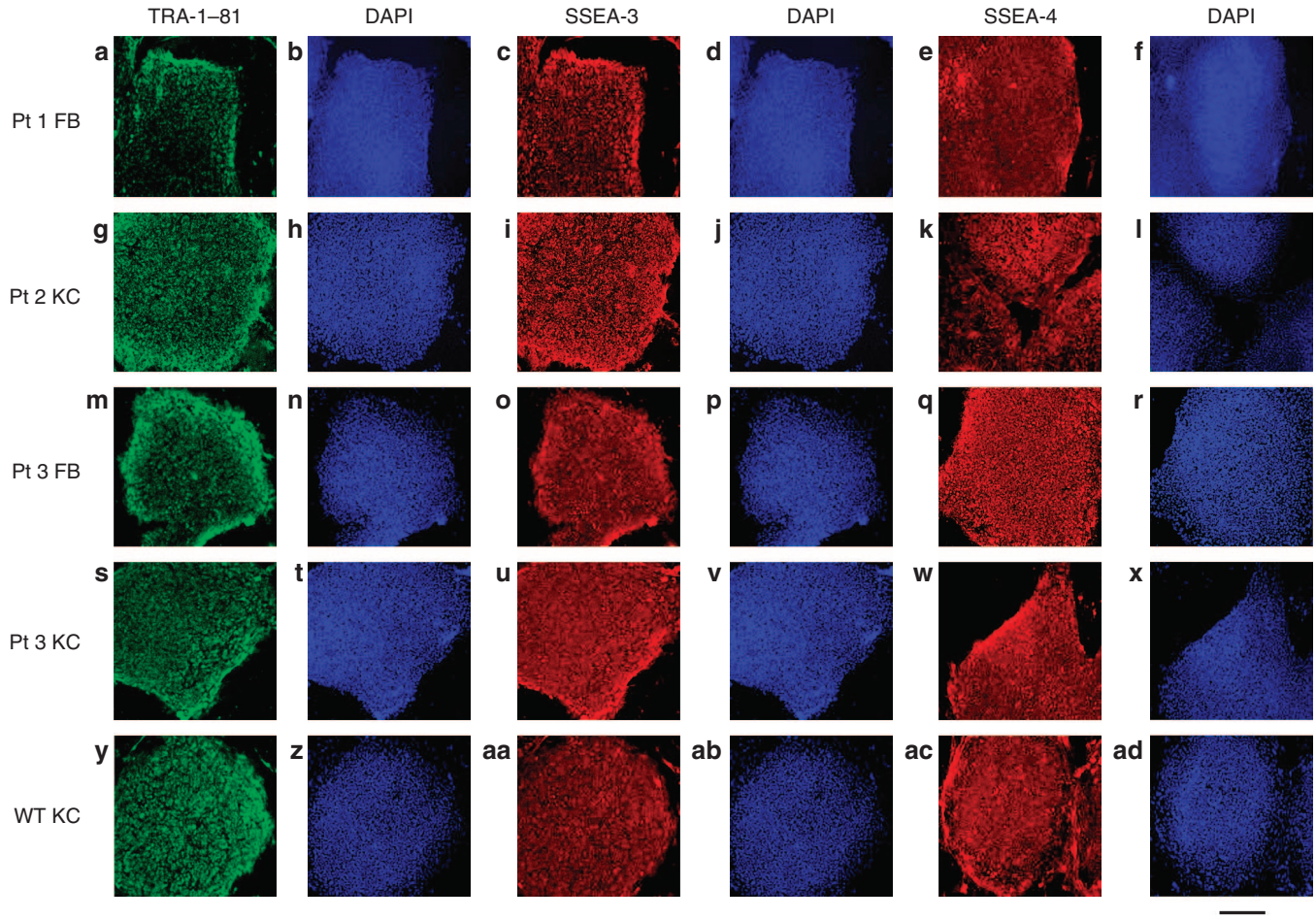

Figure 2. Protein expression profile of RDEB iPS cells. To confirm their ability to express ES cell proteins, human iPS cells derived from the skin of all RDEB patients and WT controls were immunostained with TRA-1-81 (**a**, **g**, **m**, **s**, **y**), SSEA-3 (**c**, **i**, **o**, **u**, **aa**), and SSEA-4 (**e**, **k**, **q**, **w**, **ac**). Corresponding images stained with 4,6-diamidino-2-phenylindole show nuclei of individual cells in the colonies (**b**, **h**, **n**, **t**, **z**, **d**, **j**, **p**, **v**, **ab**, **f**, **l**, **r**, **x**, **ad**). Isotype controls are shown in Supplementary Figure S8 online. DAPI, 4,6-diamidino-2-phenylindole; ES, embryonic stem; FB, fibroblast; iPS cells, induced pluripotent stem cells; KC, keratinocyte; RDEB, recessive dystrophic epidermolysis bullosa; WT, wild type. Bar = 50 μm.

hematopoietic growth factors: stem cell factor, Flt3 ligand, interleukin-3, interleukin-6, granulocyte-colony-stimulating factor, and bone morphogenetic protein 4. To define the commitment of these cells to the hematopoietic lineage, we assessed their capacity to function as colony-forming units. The colony-forming capacity is a standard measure to assess both the quality and the quantity of blood-forming stem cells and progenitor cells. The colony-forming capacity of the hematopoietic progeny of RDEB iPS cells was comparable with that of the hematopoietic progeny of WT iPS cells, and both erythroid and myeloid colonies formed (Supplementary Figure S5B–D online). In addition to quantitative measures, such as the ability to form colonies in the semisolid medium, cells with phenotypic characteristics of myeloid and erythroid progenitors were observed in equal distribution on cytospins generated from WT iPS cells and RDEB iPS cell colonies grown in a methylcellulose-enriched medium (data not shown). In aggregate, these data confirm that RDEB iPS cells can differentiate into cells with hematopoietic potential.

RDEB iPS cells differentiate into skin-like structures

With our primary focus on skin pathology relevant to RDEB subjects, we wished to identify teratoma-derived structures resembling the skin. To delineate this ectodermal-to-skin transition, we used staining with the K5 antibody to reveal epidermis-like layers of KCs. Remarkably, in WT iPS cell-derived teratomas, Col7 was expressed as a continuous band mimicking Col7 expression at the basement membrane in the normal skin (Figure 5a–d and Supplementary Figure S6A online). In contrast, and consistent with Col7 deficiency in RDEB individuals, no Col7 was detected in the skin-like structures derived from RDEB iPS cells (Figure 5e–h and Supplementary Figure S6B online). To demonstrate that RDEB iPS cells can be gene corrected with exogenous Col7 DNA, RDEB iPS cells were transfected with expression plasmid harboring the WT human *Col7* gene. Two days after transfection, the Col7 protein was expressed in gene-corrected RDEB iPS cultures similar to the pattern of Col7 expression observed in WT iPS cells (Supplementary Figure S7 online). In summary, these data suggest that WT and RDEB

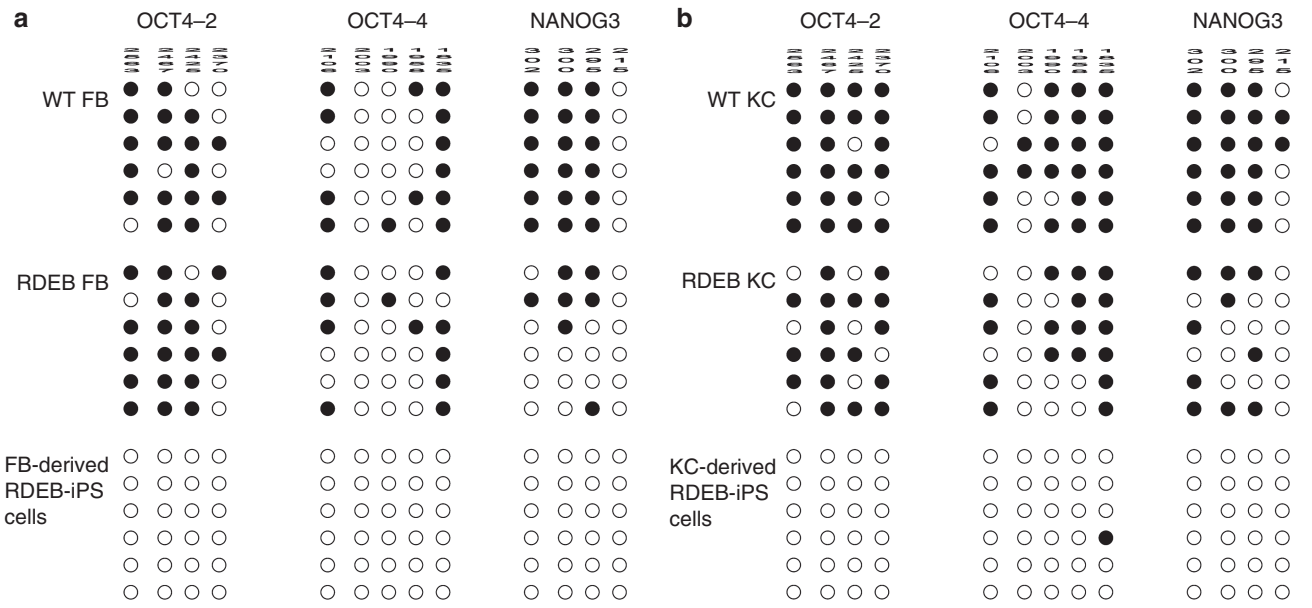

Figure 3. Epigenetic profile of RDEB iPS cells. (**a**) Bisulfite sequencing of the *OCT4* and *NANOG* promoters in WT FB, RDEB FB, and FB-derived RDEB iPS cells. (**b**) Bisulfite sequencing of the *OCT4* and *NANOG* promoters in WT KC, RDEB KC, and KC-derived RDEB iPS cells. Open circles denote unmethylated CpGs, and filled circles represent methylated CpGs. The CpG position relative to the downstream transcriptional start site is shown above each column. Sequencing reactions of specific amplicons are represented by each row of circles. FB, fibroblast; iPS cells, induced pluripotent stem cells; KC, keratinocyte; RDEB, recessive dystrophic epidermolysis bullosa; WT, wild type.

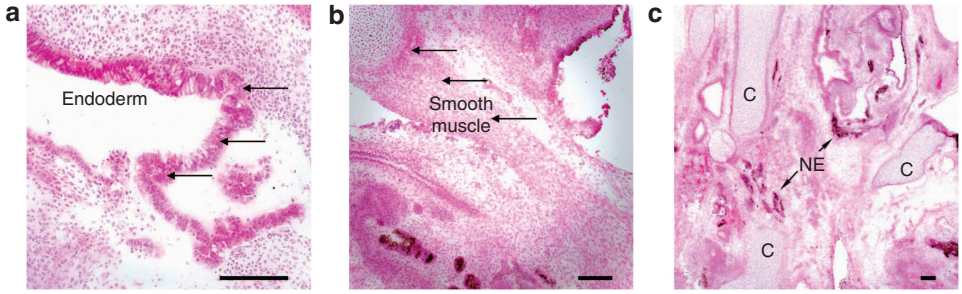

Figure 4. Nonhematopoietic differentiation of RDEB iPS cells. Histological examination of mature teratoma from immunodeficient mice injected with KC RDEB iPS cells revealed (**a**) a columnar epithelium of endodermal origin (arrows), (**b**) smooth muscle of mesodermal origin (arrows), and (**c**) melanocytes of ectodermal origin (arrows). Similar mature teratomas with contribution of ectodermal-, mesodermal-, and endodermal-derived cells formed after injection of KC WT iPS cells (data not shown). Hematoxylin-eosin stain. C, cartilage; iPS cells, induced pluripotent stem cells; KC, keratinocyte; NE, neuroectoderm; RDEB, recessive dystrophic epidermolysis bullosa; WT, wild type. Bar = 50 μm.

iPS cells can provide a robust system to study skin pathology in a patient-specific manner.

DISCUSSION

To our knowledge, these are the first data to report that autologous iPS cells can be obtained from RDEB subjects. We found that iPS cells can be generated with a similar efficacy of reprogramming using either skin FBs or KCs of individuals with RDEB. These RDEB iPS cells can be induced to differentiate into both hematopoietic and nonhematopoietic cells. Our observation that RDEB iPS cells have the capacity to form skin-like structures with no Col7 deposition underscores the potential of RDEB iPS cells in modeling RDEB in development, in uncovering clinically relevant compensatory changes triggered by the absence of Col7, and in the search for effective therapeutic interventions for RDEB patients. Relevant to this, we found that the NANOG promoter in RDEB FBs and KCs is hypomethylated. This suggests that increased proliferative signaling engaged in aberrant skin repair in individuals with RDEB could, in theory, engage cell-cycle regulation common to rapid growth, self-renewal, and pluripotency states. Finally, we found that RDEB iPS cells express Col7 after transfection of WT Col7. In the future, gene-corrected RDEB iPS cells could be used not only to generate an autologous hematopoietic graft but also to generate nonhematopoietic skin cells with the potential to treat individual skin and mucosal wounds. Independently or together, these two strategies have the potential to decrease the complications or lack of sustained improvement observed with currently used therapies and to result in improved survival and quality of life for individuals with RDEB.

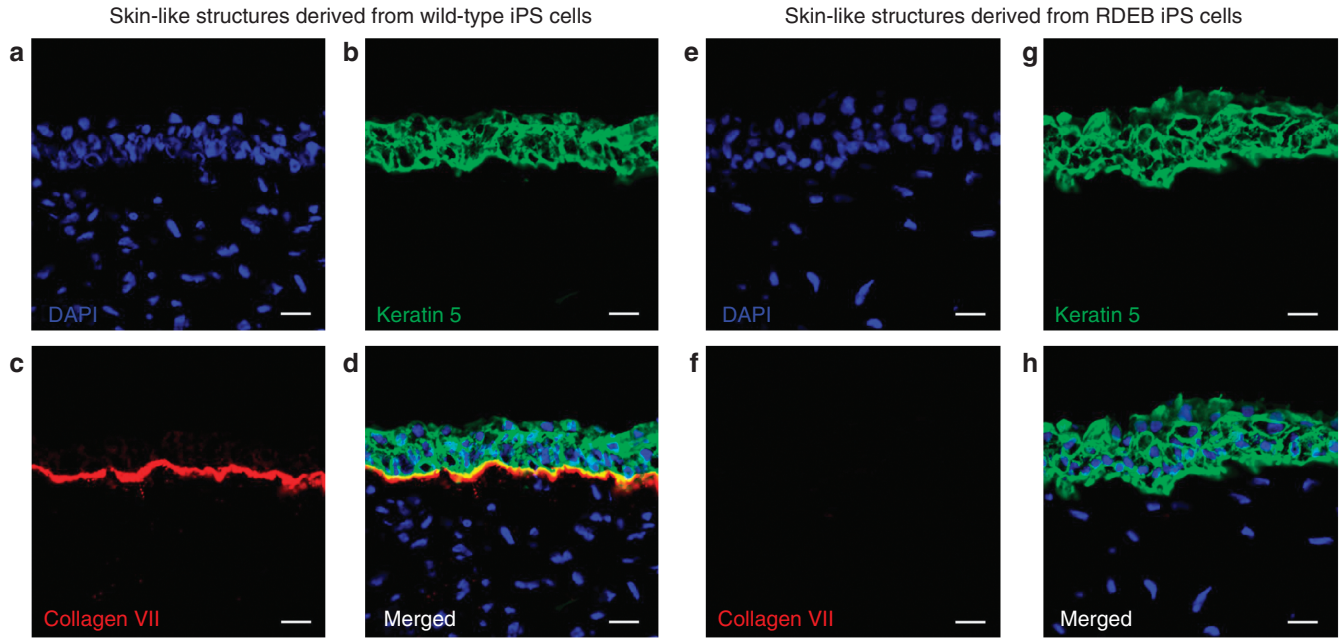

Figure 5. Skin-like structures derived from WT and RDEB iPS cells. (**a**–**d**) K5 (green) and Col7 (red) are coexpressed in WT KC iPS cell-derived teratomas. (**e**–**h**) Similar structures form in RDEB KC iPS cell-derived teratomas, yet Col7 is undetectable. DAPI, 4,6-diamidino-2-phenylindole; iPS cells, induced pluripotent stem cells; KC, keratinocyte; RDEB, recessive dystrophic epidermolysis bullosa; WT, wild type. Bar = 50 μm.

To the best of our knowledge, this is the first described human iPS cell model of an extracellular matrix disorder. As RDEB iPS cells exhibit a disease-relevant phenotype, they provide a model system to study the pathogenesis of RDEB and related disorders. When compared with studies of differentiated human cells and animal models of RDEB, iPS cell studies have the added benefit of offering the opportunity for a more extensive analysis of the cellular effects of Col7 deficiency independent of the secondary effects on systemic inflammatory responses that would occur *in vivo* because of erosions and blister formation characteristic of RDEB. As nearly all cell types can be derived from iPS cells, analysis of human RDEB iPS cells may have advantages over analysis of murine animal models that are confounded by the obvious differences in development, life span, and species-specific consequences of cellular pathology in the skin and mucosal membranes affected by Col7 deficiency. Relevant to this and in contrast to other congenital disorders, such as those affecting DNA repair genes in Fanconi's anemia (Raya *et al.*, 2009; Tulpule *et al.*, 2010), Col7 does not seem to be necessary for stem cell self-renewal and generation of iPS cells.

The complexity of RDEB, as evidenced by a plethora of biochemical events downstream of the Col7 pathology and by the varied phenotypes of human disease, is perhaps matched by the versatility and promise of reprogramming cell technology (Takahashi and Yamanaka, 2006b; Maherali *et al.*, 2008b; Park *et al.*, 2008c; Ebert *et al.*, 2009; Vierbuchen *et al.*, 2010). An unresolved question, however, is whether the potential risks of iPS cell-derived therapy, such as those related to the following: (1) the genotoxicity of reprogramming using embryonic stem cell-specific transcription factors and the methods by which gene augmentation of mutated genes is accomplished (e.g., viral vector delivery) (Kohn *et al.*, 2003); (2) fidelity of acquisition and maintenance of pluripotent phenotype and lineage-specific cell fates; and (3) cell purity of the on-demand differentiated iPS cell progeny in the absence of undifferentiated and potentially tumorigenic iPS cells, can be minimized or avoided altogether.

Nevertheless, even at present, RDEB iPS cells offer a model of RDEB disease that is to our knowledge previously unreported and that may help to understand the pathogenic cascades underlying the inherent complexity of RDEB. These insights, and the use of the cellular progeny of gene-corrected autologous RDEB iPS cells, present an opportunity for future clinical translation in a manner that may preclude the immunological complications of allogeneic transplantation.

MATERIALS AND METHODS

Patients

After obtaining written, informed consent as approved by the Institutional Review Board of the Human Subjects Committee at the University of Minnesota, skin or marrow cells were collected from healthy volunteers and from individuals with RDEB. All procedures adhered to the Helsinki Guidelines.

iPS cells

Detailed methods describing the isolation of FBs and KCs are included as Supplementary Materials online. For induction of iPS cells, four reprogramming factors, namely OCT4, SOX2, KLF4, and c-MYC were used to produce retroviral supernatants. Each of the viral supernatants was produced by transfecting 293T/17 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with three plasmids: one cargo plasmid (containing the reprogramming gene), a plasmid expressing the *VSV-G* envelope gene, and a helper plasmid with the retroviral *Gag/Pol* gene. The plasmids were obtained from Addgene (Cambridge, MA). In all, 48 to 72 hours after transfection,

viral supernatants were harvested, centrifuged at $400 \times g$ for 15 minutes, and filtered through a 0.45-μm filter.

Approximately 50,000 KCs or 100,000 FBs per well of a 6-well plate were plated and infected with a 1:1:1:1 mix of retroviral supernatants of pMIG containing OCT4, SOX2, KLF4, and c-MYC in the presence of 5 μg ml^{-1} of protamine sulfate. Transduction consisted of a 45-minute spinfection at 1,800 r.p.m. at room temperature; supernatants were left in contact with the cells for 24 hours at 37 °C and 5% CO_2. The next day, cells were spinfected for a second time. Cells were placed in plates coated using a Coating Matrix Kit (Cascade Biologics, Portland, OR) and maintained in the KC medium (EpiLife Medium, Cascade Biologics: 0.06 mM calcium chloride, plus human KC growth supplement) at 37 °C, 5% CO_2. Five days later, cells were trypsinized and seeded onto feeder layers of irradiated CF1 MEFs. After 24 hours, the medium was changed to human embryonic stem cell medium, consisting of DMEM/F12 (Invitrogen) supplemented with 10% KnockOut Serum Replacement (Invitrogen), 2 mM GlutaMAX (Invitrogen), 50 μM 2-mercaptoethanol (Invitrogen), 1 × nonessential amino acids (Invitrogen), 50 Units ml^{-1} penicillin, 50 mg ml^{-1} streptomycin, and 10 ng ml^{-1} basic FB growth factor (R&D Systems, Minneapolis, MN). Cultures were maintained at 37 °C, 5% CO_2, with daily medium changes. Starting 1 week after plating onto MEFs, the medium was supplemented with 1 μM PD0325901 and 1 μM CT 99021 (both from Stemgent, San Diego, CA) for 1 week. Colonies were picked based on morphology, 30–60 days after the initial infection.

We identified iPS cell colonies using live staining with TRA-1-60 antibody (1:400, Millipore, Billerica, MA) and secondary antibody Alexa 488-conjugated anti-mouse IgM (1:400, Invitrogen) diluted in the hES medium, and added into the culture plate. TRA-1-60 + colonies were identified under a fluorescence microscope (Leica DMI6000B, Leica, Bannockburn, IL). To confirm that iPS cells were not contaminated with donor cells, competitive PCR analysis of variable tandem repeat regions was performed as described previously (Scharf *et al.*, 1995).

To confirm their cellular phenotype, iPS cells were fixed with 4% paraformaldehyde for 20 minutes. If nuclear permeation was required, cells were treated with 0.2% TritonX (Sigma-Aldrich, St Louis, MO) in phosphate-buffered saline (PBS) for 30 minutes, blocked in 3% bovine serum albumin in PBS for 2 hours, and incubated with the primary antibody overnight at 4 °C. Antibodies targeting the following antigens were used: TRA1-60 (MAB4360, 1:400), TRA1-81 (MAB4381, 1:400), stage-specific embryonic antigen-4 (MAB4304, 1:200), and stage-specific embryonic antigen-3 (MAB-4303, 1:200), all from Chemicon (Billerica, MA), OCT3/4 (AB27985, 1:200) from Abcam (Cambridge, MA), and NANOG (EB068601:100) from Everest (Upper Heyford, Oxfordshire, UK). Cells were incubated with secondary Alexa Fluor Series antibodies (all 1:500, Invitrogen) for 2 hours at room temperature and then with DAPI (4,6-diamidino-2-phenylindole) (1 μg ml^{-1}, Invitrogen) for 10 minutes. Images were examined using a Leica DMI6000B microscope equipped with Q-Imaging Retiga 2000R Camera and Q-Capture software (QImaging, Surrey, BC, Canada). Direct alkaline phosphatase activity was analyzed as per the manufacturer's recommendations (Millipore).

Reverse transcriptase-PCR

RNA was isolated using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) and treated with TURBO DNA-free (Ambion, Austin, TX). First-strand cDNA was synthesized using a Superscript III First-Strand Synthesis SuperMix for quantitative reverse transcriptase-PCR (Invitrogen). Reverse transcriptase-PCR was performed using TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Carslbad, CA) as per the manufacturer's protocol.

TaqMan gene expression assays used were Col7a1 (Hs01574733_g1), Col17 (Hs00166711_m1), Ker1 (Hs00196158_m1), Ker15 (Hs00267035_m1), POU5F1 Hs 00999634_gH; SOX2 Hs 00602736_s1; NANOG Hs 02387400_g1; KLF4 Hs 00358836_m1; MYC Hs 00153408_m1; LIN28 Hs 00702808_s1; DNMT3 Hs 01003405_m1, with GAPDH (Hs99999905_m1) used as an endogenous control. Expression levels were measured in duplicate. For genes with expression below the C_T fluorescence threshold, C_T was set at 40 to calculate the relative expression. Analysis was performed using an ABI PRISM 7500 sequence detection system (Applied Biosystems).

Bisulfite genomic sequencing

Genomic DNA was isolated using a PureLink Genomic DNA Mini Kit (Invitrogen). Bisulfite treatment was carried out using an EpiTect Bisulfite kit (Qiagen, Valencia, CA). Converted DNA was PCR amplified using OCT4- and NANOG-specific primer sets (Freberg *et al.*, 2007). PCR products were gel purified using a PureLink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen), and cloned into bacteria using the TOPO TA Cloning Kit for Sequencing (Invitrogen).

Differentiation of iPS cells

Detailed methods describing isolation of hematopoietic differentiation of iPS cells are included as Supplementary Materials online. For teratoma formation, NOG young adult mice were injected with 1 million cells resuspended in a mixture of DMEM/F12, Matrigel (BD Biosciences, San Jose, CA), and collagen (ratio 2:1:1, 40 μl per mouse) into the right quadriceps muscle. Tumors were harvested in 4–8 weeks and cryopreserved at −80 °C in optimal cutting temperature medium (Sakura Finetek USA, Torrance, CA). Sections of 6-μm-thickness were cut and mounted on Superfrost Plus glass slides (Thermo Fisher Scientific, Waltham, MA). Tissues were then fixed in acetone for 5 minutes at room temperature. After rehydrating the samples in PBS for 5 minutes, blocking solution of 10% normal donkey serum was applied for 1 hour at room temperature. The tissues were then incubated with mouse anti-human Col7 antibody (1:250, BD Biosciences), and rabbit anti-human K5 antibody (1:600, Covance, Emeryville, CA) and then incubated with secondary antibodies, anti-mouse Cy3 (1:500, Jackson ImmunoResearch, West Grove, PA), and goat anti-rabbit Alexa Fluor 488 (1:800, Invitrogen). Tissues were rinsed three times with PBS and coverslipped with a hardset mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Optical parameters used for light microscopy are included as Supplementary Materials online.

Correction of RDEB iPS cells

The COL7A1-COL7A1 expression cassette, in which the full-length COL7A1 cDNA expression is driven by a short COL7A1 promoter (Titeux *et al.*, 2010), was cloned into the pEGFP-1 plasmid backbone (Clontech, Mountain View, CA) as folows: (1) pEGFP-1 was digested by *HindIII* and *NotI* to excise the enhanced green fluorescent protein (EGFP) open-reading frame, (2) the

pCMS-COL7A1-COL7A1 retroviral backbone vector (Titeux *et al.*, 2010) was digested by *HindIII* and *NotI* to release the COL7A1–COL7A1 expression cassette, and (3) pEGFP-1 (Δ-EGFP) and 10 kb COL7A1–COL7A1 fragments were ligated to generate the pCOL7A1-COL7A1-pA vector (13.4 kb). RDEB iPS cells were nucleofected with this Col7 cDNA using the Human Stem Cell Nucleofector Kit 1 (program A-023, Amaxa/Lonza, Walkersville, MD) with 1.2 mg of DNA per 1 million cells. Nucleofected cells were plated in AggreWell plates (StemCell Technologies, Vancouver, BC, Canada). After 48 hours, embryoid bodies formed and were transferred to four-chambered glass slides coated with Matrigel. To assess Col7 expression 72 hours after nucleofection, cells were fixed in a 1:1 ratio of ice-cold methanol/acetone for 7 minutes, washed with 1 × PBS, blocked with 10% normal donkey serum for 1 hour, and stained with primary Col7 antibody (1:250, BD Biosciences) for 2 hours at room temperature. Slides were washed and secondary antibody donkey anti-mouse cy3 (1:500, Jackson Immunoresearch) was applied for 1 hour. DAPI was added, and slides were imaged by confocal microscopy using Olympus BX61 FluoView 500 confocal microscope and FluoView software (Olympus, Center Valley, PA).

Data analysis

Differences between measurements were evaluated using Student's *t*-test, with *P*-values <0.05 considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Dr Gerry O'Sullivan for examination of teratomas, and Marianna Wong, Jess Lorenz, Trevor Keyler, and Pavlina Chuntova for preparing laboratory data. We are grateful to Dr John McGrath for stimulating discussions on developing therapies for RDEB. We would like to acknowledge the use of confocal microscope made available through an NCRR Shared Instrumentation Grant (#1 S10 RR16851). This study was supported in part by grants from DEBRA International, Epidermolysis Bullosa Research Fund, University of Minnesota Academic Health Center, and by the Liao Family Epidermolysis Bullosa Fund, Sarah Rose Mooreland Epidermolysis Bullosa Fund, and Children's Cancer Research Fund, Minneapolis, Minnesota.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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