Long-Term Type VII Collagen Restoration to Human Epidermolysis Bullosa Skin Tissue

Zurab Siprashvili, Ngon T. Nguyen, Maria Y. Bezchinsky, M. Peter Marinkovich,
Alfred T. Lane, and Paul A. Khavari

Abstract

In spite of advances in the molecular diagnosis of recessive dystrophic epidermolysis bullosa (RDEB), an inherited blistering disease due to a deficiency of type VII collagen at the basement membrane zone (BMZ) of stratified epithelium, current therapy is limited to supportive palliation. Gene delivery has shown promise in short-term experiments; however, its long-term sustainability through multiple turnover cycles in human tissue has awaited confirmation. To characterize approaches for long-term genetic correction, retroviral vectors were constructed containing long terminal repeat-driven full-length and epitope-tagged *COL7A1* cDNA and evaluated for durability of type VII collagen expression and function in RDEB skin tissue regenerated on immune-deficient mice. Type VII collagen expression was maintained for 1 year *in vivo*, or over 12 epidermal turnover cycles, with no abnormalities in skin morphology or self-renewal. Type VII collagen restoration led to correction of RDEB disease features, including reestablishment of anchoring fibrils at the BMZ. This approach confirms durably corrective and noninjurious gene delivery to long-lived epidermal progenitors and provides the foundation for a human clinical trial of *ex vivo* gene delivery in RDEB.

Introduction

THE ARRAY OF BLISTERING SKIN DISEASES, collectively known as epidermolysis bullosa (EB), represents prototypes of monogenic cutaneous skin diseases (Eady and Dunnill, 1994; Korge and Krieg, 1996; Paller, 1996; Uitto and Pulkkinen, 1996). Molecular alterations in a number of specific genes responsible for EB have been well characterized (Marinkovich, 1993; Korge and Krieg, 1996; Paller, 1996; Uitto and Pulkkinen, 1996). Malfunction in any of their corresponding proteins impairs epidermal adhesion and results in epidermal fragility and blistering. The severe Hallopeau–Siemens subtype of recessive dystrophic epidermolysis bullosa (RDEB) is caused by recessive mutations in the type VII collagen gene (*COL7A1*) that result in abnormal or reduced type VII collagen protein at the basement membrane zone (BMZ) of the skin (Heagerty *et al.*, 1986; Leigh *et al.*, 1988). Subjects with RDEB develop painful blisters and wounds on the skin and mucous membranes, leading to a shortened life expectancy due to infection, organ failure, or squamous cell carcinoma (SCC). Preclinical RDEB studies have suggested various treatment strategies for the disease, such as direct virus-based (Woodley *et al.*, 2004b), protein-based (Woodley *et al.*, 2004a; Remington

et al., 2009), and cell-based therapies including bone marrow-derived cells (Tolar *et al.*, 2009) and genetically modified keratinocytes (Chen *et al.*, 2002; Ortiz-Urda *et al.*, 2002; Gache *et al.*, 2004) or fibroblasts (Ortiz-Urda *et al.*, 2003; Woodley *et al.*, 2003; Kern *et al.*, 2009). The promise suggested by this body of preclinical research points to the emergence of new treatments for RDEB beyond currently available palliative wound care.

Among such promising treatments is *ex vivo* gene therapy, an approach that requires targeting RDEB progenitors for durable gene correction. In a related EB subtype, junctional epidermolysis bullosa (JEB), a single case of successful correction has been clinically reported (Mavilio *et al.*, 2006). This lone success used an *ex vivo* approach involving the transplantation of cultured epidermal grafts prepared from retrovirally transduced autologous keratinocytes. Efficacious gene therapy for JEB was an extraordinary achievement and suggests that a similar *ex vivo* approach can be developed for RDEB. However, several issues need to be considered for gene therapy of RDEB. First, the human *COL7A1* mRNA is approximately 9.2 kb in length and poses technical challenges for effective gene delivery (Christiano *et al.*, 1994a,b). Second, duration of *COL7A1*

gene expression as well as its long-term effect on human epidermal homeostasis and immune surveillance are currently unknown.

One preclinical model to study skin homeostasis *in vivo* uses severe combined immunodeficiency (SCID) mice as a recipient of a transplantable human skin equivalent. This model has been used to confirm that the time period of complete human epidermal turnover is 3 to 4 weeks (Robbins *et al.*, 2001). To address the durability of transgene expression and to properly characterize biological function of the exogenous type VII collagen, several modifications of this model are required. First, reintroduced type VII collagen must be distinguishable from the endogenous protein present as a structural component of the underlying dermis. During preparation of the dermal support, epitopes of the endogenous type VII collagen are retained, in spite of partial or full degradation of the native protein. These epitopes can be detected in postgrafting experiments, interfering with identification of the type VII collagen produced by genetically modified keratinocytes. This problem can be solved by using either epitope-tagged type VII collagen, or nonhuman dermis lacking epitopes that cross-react with human type VII collagen. The cross-species model is advantageous in short experiments, but is not suitable for long-term studies because of its fragility.

Here we address these issues to demonstrate that full-length type VII collagen can be secreted by retrovirally transduced human keratinocytes for 1 year *in vivo* without any noticeable tissue toxicity. In one set of experiments, epitope-tagged type VII collagen was developed and used to establish the durability of protein expression in grafted skin. In a second set of experiments, porcine dermis was used to assess delivery of native human type VII collagen in regenerated skin *in vivo* via corrected human RDEB cells. Long-term type VII collagen reexpression leads to sustained correction of central RDEB disease features. These preclinical studies provided the foundation for a human phase I clinical trial of *ex vivo* gene delivery in RDEB approved by U.S. Food and Drug Administration (BB-IND-13708).

Materials and Methods

Vector construction

Recombinant pLZRS-COL7A1 plasmid was generated on the basis of pLZRS-LacZ(A) (Kinsella and Nolan, 1996). First, the full-length COL7A1 gene (9226 bp) containing the open reading frame (ORF) and a 3' untranslated region (UTR) was subcloned from pcDNA3-COL7A1 plasmid into the pCEP4 vector (Invitrogen, Carlsbad, CA), using *HindIII* and *NotI* sites. The 3' UTR region was removed by PCR-based mutagenesis and the primer pair SapF (5'-CTCTC ATGCAGAGGAGGAAGA) and C7-C (5'-TTTTCGCGC CGCGAATTCTCAGTCCTGGCAGTACCTG). A DNA fragment containing the C-terminal part of COL7A1 and lacking the 3' UTR region was ligated back into pCEP4-COL7A1. This vector, pCEP4-COL7A1Δ3', was used as a source of the full-length COL7A1 gene. A *HindIII*-*NotI* fragment containing type VII collagen-coding UTR-reduced cDNA (8832 bp) was cloned from the pCEP4-COL7A1Δ3' vector into pLZRS-LacZ(A), replacing the *lacZ* gene with COL7A1 to create pLZRS-COL7A1. Last, site-specific mutagenesis was used to remove ORFs within the *gag* and *pol*

genes of the packaging signal. The primers used were as follows:

GAG1: 5'-GTATTTGTCTGAAAATTTGGGCCCGGGCCA GACTG
 GAG2: 5'-CAGTCTGGCCCGGGCCCAAAATTTTCAGAC AAATAC
 GIN1: 5'-TCACAACCAGTCGGTAGTTGTCAAGAAGAG ACGTTGG
 GIN2: 5'-CCAACGTCTCTTCTTGACAACCTACCGACTG GTTGTGA
 POL1: 5'-CTTCCCTGACCCTGACTTGACAAGAGTTAC TAAC
 POL2: 5'-GTTAGTAAC TCTTGTCAAGTCAGGGTCAGG GAAG

The ORFs within the *gag* and *pol* regions were mutated by introducing A1476T, A1567T, and A1968T substitutions to generate an ORF-reduced extended packaging sequence. The final pLZRS-COL7A1 vector was fully sequenced from the 5' long terminal repeat (LTR) to the 3' LTR and used for further virus production steps.

The HACOL7A1 gene was generated by site-directed mutagenesis and overlapping PCR techniques. A 3×HA tag was introduced between the type VII collagen signal peptide and Gln-24, using the following primers:

HA-F: 5'-TATCCCTATGACGTCCCTGATTATGCAGGA AGCTATCCCTACGACGTGCCCGATTACGCCCAGC ACAGGGAGAGAGTGAC
 Bcl-R: 5'-CCGTCTGTGATCAGGATGTCAG
 C7NH-F: 5'-TTTTCGCTAGCAAGCTTACCATGACGCTGC GGCTTCTGGT
 HA-R: 5'-GCTTCCTGCATAATCAGGGACGTCATAGGG ATAGCCAGCATAGTCAGGCACATCGTATGGGTAG GCTCGCACTCGGGGCG

A DNA fragment containing COL7A1 flanked with N-terminal triple hemagglutinin (HA) tag was ligated into *NheI*- and *BclI*-digested pCEP4-COL7A1Δ3' vector. A *HindIII*-*NotI* fragment containing HA-tagged type VII collagen-coding cDNA (8948 bp) was recloned from pCEP4-HACOL7A1 vector into pLZRS-LacZ(A), replacing the *lacZ* gene with HACOL7A1 to create pLZRS-HACOL7A1. This vector was sequenced to confirm HA tag integrity and used for subsequent virus production steps.

The control reporter retroviral vector pLZRS-LY contained firefly luciferase and the enhanced yellow fluorescent protein (EYFP) fusion gene under the control of the LTR promoter. First, luciferase from pGL3-Basic (Promega, Madison, WI) was fused with the EYFP-encoding gene from pEYFP (Clontech, Mountain View, CA) via the sequence 5'-GTGGAGGGGTACCGGCGGCGGCGGAGGCGT, encoding the TrpArgGlyThr₅GlyVal polypeptide linker, between these two proteins. The DNA fragment containing the LY gene was flanked with 5' *HindIII* and 3' *BamHI*, *EcoRV*, *EcoRI*, and *NotI* sites and cloned into *HindIII*-*NotI* sites of the pcDNA3.1 vector (Invitrogen) to produce the pcDNA3.1-LY plasmid. Next, the *BamHI*-*MscI* fragment containing the foot-and-mouth disease virus internal ribosomal entry site and the hygromycin resistance gene was recloned from pMONO-hygro-mcs (Invivogen, San Diego, CA) into *BamHI*-*EcoRV* sites of the pcDNA3.1-LY plasmid. Last, the *AflIII*-*NotI* fragment from pcDNA3.1-LY was removed and cloned

into blunted *Bam*HI–*Not*I sites of the pLZRS retrovirus to create the final pLZRS-LY vector.

Retrovirus production

The amphotropic retroviral Phoenix packaging cell line (Kinsella and Nolan, 1996) was plated on a 100-mm tissue culture dish and the next day, when cells reach at least 40% confluence, they were transfected with 10 μ g of pLZRS-COL7A1, pLZRS-HACOL7A1, or pLZRS-LY plasmid, using FuGENE 6 reagent (Roche Applied Sciences, Indianapolis, IN) in accordance with the manufacturer's recommendations. Forty-eight hours posttransfection puromycin selection (1 μ g/ml) began and continued for an additional 5 days. For the virus collection, cells were split into 150-mm tissue culture dishes and grown until confluent. The plates were then transferred to a 32°C incubator and after 24 hr viral supernatant was collected over 3 to 4 consecutive days. Each collection was passed through a 0.45- μ m (pore size) filter unit (Nalgene, Rochester, NY), and aliquots were stored at –80°C. To confirm recombinant construct functionality, viral supernatants containing Ampho-LZRS-COL7A1, Ampho-LZRS-HACOL7A1, or Ampho-LZRS-LY were used to infect COL7A1-negative RDEB keratinocytes. Type VII collagen expression was evaluated by Western blot analyses and immunofluorescence (IF), using antibodies against either type VII collagen or the HA tag.

To produce xenotropic virus, Ampho-LZRS-COL7A1, Ampho-LZRS-HACOL7A1, or Ampho-LZRS-LY virus was used to infect the xenotropic packaging cell line PG13, using 6-well tissue culture plates as described below (Miller *et al.*, 1991). Forty-eight hours postinfection the cells were trypsinized and transferred into 48-well plates at limiting dilution. The next day, wells containing single cells were marked and grown until the single colonies reached 2–3 mm in size. Individual PG13-LZRS-LY clones were analyzed by fluorescence microscopy and the wells containing uniform green staining were chosen for further virus production. The supernatants from wells containing the progeny of PG13-LZRS-COL7A1 or PG13-LZRS-HACOL7A1 single-cell populations were collected and used for RNA purification with RNeasy reagent (Invitrogen) according to the manufacturer's recommendations. To quantify the viral supernatant titer, RT-PCR was performed with provirus-specific primers for the 5' region (LZRS2F, 5'-TGGATACACGCCGCC ACGTG; and C7-R1, 5'-GCTCCTCAGGGTCAGCATT) and the 3' region (SapF, 5'-CTCTCATGCAGAGGAGGAAGA; and LZRS2R, 5'-ATCGTCGACCACTGTGCTGG).

Individual clones positive in the RT-PCR analysis were trypsinized and transferred first into 12-well plates and then propagated in 6-well culture dishes. The gibbon ape leukemia virus (GALV)-pseudotyped LZRS-COL7A1, LZRS-HACOL7A1, and LZRS-LY viruses were harvested after 24 hr at 32°C and used to infect COL7A1-negative RDEB keratinocytes in order to evaluate type VII collagen production in these cells. The PG13 clones with the highest production level of type VII collagen in RDEB cells were used in further experiments.

Primary keratinocyte preparation

Wild-type keratinocyte cells were isolated from neonatal foreskin, whereas autologous primary RDEB keratinocytes were isolated from patient skin samples. The latter was ob-

tained as a 2×8 mm punch biopsy submerged in 35 ml of biopsy transport medium 50/50A containing keratinocyte growth medium 50/50V and amphotericin B (0.5 μ g/ml) (Invitrogen). Medium 50/50V contained 50% medium 154 (Invitrogen) supplemented with HKGS (0.2% bovine pituitary extract [BPE], bovine insulin [5 μ g/ml], hydrocortisone [0.18 μ g/ml], bovine transferrin [5 μ g/ml], human epidermal growth factor [0.2 ng/ml]) and 50% keratinocyte SFM (KSFM; Invitrogen) supplemented with recombinant human EGF_{1–53} (0.1–0.2 ng/ml), BPE (20–30 μ g/ml), and AV100 antibiotic solution with amikacin (30 μ g/ml; Sigma-Aldrich, St. Louis, MO) and vancomycin (20 μ g/ml; Sigma-Aldrich).

To separate the epidermis from the dermis, each skin sample was cut into four pieces and placed in 10 ml of phosphate-buffered saline (PBS) containing dispase (25 caseinolytic units/ml; BD Biosciences, San Jose, CA) for 12 hr at 4°C. The next day, the epidermis was carefully peeled off the dermis and placed in trypsin-EDTA (Invitrogen) solution at 37°C for 15–20 min. The trypsin was quenched by adding equal volumes of a defined trypsin inhibitor (DTI, 0.006% soybean trypsin inhibitor; Invitrogen) and then spun down at 1200 rpm to pellet the keratinocytes. Cells were washed once with PBS and plated on 10-cm dishes coated with collagen (29 mg/ml; Cohesion Technologies, Palo Alto, CA) in keratinocyte growth 50/50V medium. After 4–6 days, or when keratinocytes reached 60–70% confluence, cells were trypsinized and plated in 10-cm dishes for infection at 25–35% confluence.

Cell infection

Before infection, cells were plated in 6-well or 100-mm culture plates such that they were 30% confluent 24 hr later; virus was used at 3 and 12 ml for 6-well and 100-mm plates, respectively. For infection, the viral stock was thawed at 37°C, supplemented with Polybrene (5–8 μ g/ml; Sigma-Aldrich), and incubated with cells for 1 hr at 1250 rpm and 32°C at a multiplicity of infection (MOI) of 10–15. Infected cells were then washed with PBS and substituted with fresh growth medium (Dulbecco's modified Eagle's medium [DMEM] for Phoenix and 50/50V for keratinocytes).

Type VII collagen expression analysis

Type VII collagen expression analysis was performed 48 hr after infection of RDEB or wild-type keratinocytes. Cells were divided into duplicate cultures, one for propagation and the other for verification of type VII collagen secretion. The verification culture in 6-well plates was grown to 60–70% confluence, and then the medium was replaced with 50/50V supplemented with ascorbic acid (100 μ g/ml; Sigma-Aldrich) for at least 24 hr. The supernatant from these wells was then analyzed directly or concentrated 50-fold with Microcon filtration units (Amicon, Bedford, MA). Type VII collagen secretion was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by immunoblotting with anti-type VII collagen polyclonal antibody FNC1 (raised against the NC1 domain of the type VII collagen) (Ortiz-Urda *et al.*, 2005), or with monoclonal antibody NP185 (Chemicon, Temecula, CA). HA–type VII collagen was detected with HA monoclonal antibody (cone HA.11; Covance, Emeryville, CA). Corrected subject cells were used for grafting experiments if they expressed

levels of type VII collagen greater than or equal to that observed in wild-type keratinocytes. Negative controls included mock-infected RDEB subject keratinocytes that lack type VII collagen.

Transduction efficiency was quantitated by immunofluorescence in 4-well chamber slides. Briefly, 5×10^3 transduced cells were seeded into 4-well chamber slides and analyzed the next day by IF, using anti-type VII collagen monoclonal antibody NP185 (Chemicon) or the FNC1 polyclonal antibody. HA-type VII collagen was detected with the anti-HA monoclonal (clone HA.11; Covance) or polyclonal (clone Y-11; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. The virus transduction efficiency (VTE) was calculated as the ratio of the number of type VII collagen-positive cells to total nuclei.

For IF analyses of skin grafts, antibodies to keratin-15, keratin-10, keratin-14, and loricrin were obtained from Covance. Desmoglein-3 was obtained from Zymed (South San Francisco, CA). Mouse monoclonal antibody to the HA tag was obtained from Covance, and rat monoclonal antibody to the HA tag was obtained from Roche (Indianapolis, IN).

Skin graft preparation

Skin grafts were prepared with either epidermal sheets or composite skin grafts. Briefly, 100% confluent 6-well plates of keratinocytes were supplemented with sterile 1.2 mM CaCl_2 solution (Invitrogen) and left for at least for 12 hr to facilitate epidermal sheet formation. The next day, medium was removed, epidermal sheets were washed with PBS, and HBSS containing dispase at 25 caseinolytic units/ml (BD Biosciences) was added at room temperature to initiate sheet detachment. The detached sheet was then washed at least four times with 2 ml of PBS to remove residual dispase. The outer layer of a TELFA nonadherent dressing (Tyco Healthcare/Kendall, Mansfield, MA), precut into about 2×2 cm pieces, or the same size nonadherent petrolatum gauze (Tyco Healthcare/Kendall), was carefully placed on the top of the epidermal sheet, which was lifted off the plate by the corners. The epidermal sheet was placed directly onto dermis with the cells facing the dermis and grafted as described below.

For composite skin graft production, transduced keratinocytes were plated in 100-mm plates and grown to 60–70% confluence with 50/50V growth medium. On the day of composite skin graft preparation, human (AlloDerm; LifeCell, Branchburg, NJ) or porcine dermis was cut into about 2×2 cm pieces and dried under aseptic conditions at the bottom of the 6-well tissue culture plate for 2 hr. At least 5×10^6 cells were resuspended in a maximal volume of 150 μl of 50/50V and placed in the middle of the dermis to avoid spillage of the cells onto the plate surface. After another 2 hr, wells were filled with 2.5 ml of keratinocyte growth medium and allowed to grow further for at least 48 hr or until cells were visibly growing over the edges of the dermis. Next, the composite skin graft was removed from the plate with forceps touching only one side of the graft and used immediately for transplantation as described below.

Animal experiments

Animal and human tissue studies were approved by corresponding institutional review boards. Mouse grafting was performed as previously described (Choate *et al.*, 1996b). Briefly, adult C.B-17-*scid/scid* mice were anesthetized by

intraperitoneal injection of a mixture of ketamine (70 mg/kg), xylazine (15 mg/kg), and acepromazine (2 mg/kg). After shaving the hair from the mouse flank, a rectangular region of mouse skin (approximately 1.6×1.4 cm) was removed with a scalpel and the dermis (porcine or AlloDerm) was sutured to the mouse skin. An epidermal sheet was then placed on the top of the dermis and also secured to the dermis with sutures (Ethicon, Johnson & Johnson, Somerville, NJ), minimizing sheet movement against the dermis. For composite graft transplantation, a full composite graft was sutured to the mouse flank in a manner similar to the process described for dermis attachment. Both dermal and epidermal components were kept moist throughout the transplantation procedure. Nonadherent dressing (TELFA; Tyco Healthcare/Kendall) was cut into 2×2 cm squares and placed on top of the graft. Next, Tegaderm (3M Health Care, St. Paul, MN) dressing was wrapped around the mouse and then covered with a Coverlet adhesive dressing (BSN Medical, Charlotte, NC). Last, a double layer of CoFlex (Andover Healthcare, Salisbury, MA) was wrapped around the mouse. The dressing was removed 9–12 days postgrafting and grafts were then further characterized. Control mice that received uncorrected RDEB skin grafts were often rewrapped with CoFlex (Andover Healthcare) and small transparent bubblehead chambers were placed over the graft to prevent deepithelialization of the fragile graft.

For *in vivo* bioluminescence studies, luciferase expression in skin grafts was analyzed with an *in vivo* imaging system (IVIS; Xenogen/Caliper Life Sciences, Hopkinton, MA). Images representing *in vivo* bioluminescent signal were obtained over 1- to 5-min integration times and were superimposed on gray-scale reference images of the animals obtained under weak illumination; total photon emission (photons per second) from defined regions was quantified with Living Image 2.5 (Xenogen/Caliper Life Sciences).

Results

Viral constructs

Despite numerous attempts at nonviral gene delivery in RDEB and other skin disorders (Ortiz-Urda *et al.*, 2002, 2003), viruses represent the most efficient method for introducing genetic material into primary human keratinocytes (Deng *et al.*, 1998; Di Nunzio *et al.*, 2008). Among Moloney murine leukemia virus (MoMLV)-based retroviruses, pLZRS has been used for its stable episomal maintenance within packaging cell lines, and its high production capacity and helper virus-free features (Kinsella and Nolan, 1996). On the basis of these characteristics, we designed expression vectors derived from pLZRS containing both wild-type and epitope-tagged cDNA from human COL7A1 (Fig. 1A). To minimize alternative translational events from the putative upstream open reading frames (ORFs) within the Ψ extended packaging sequence, all ATG sites were inactivated by site-specific mutagenesis. The resulting ORF-reduced LZRS vector was designated LZRS-E and used for construction of the target COL7A1 delivery viral vector.

The triple N-terminal human influenza hemagglutinin (HA) tag was placed between positions A23 and Q24 of type VII collagen, separating the first 23 amino acids of signal peptide from the full-length polypeptide. The amphotropic retroviruses Ampho-COL7A1 (wild-type) and Ampho-

FIG. 1. Retroviral delivery of type VII collagen into primary keratinocytes. **(A)** Retroviral constructs used in this study. LZRSE, MoMLV retroviral vector containing ORF-reduced packaging signal; COL7A1/HACOL7A1, wild-type/HA-tagged type VII collagen; Ψ , extended retroviral packaging sequence; Ψ' , ORF-reduced extended retrovirus packaging signal. **(B)** Wild-type keratinocytes infected with Ampho-COL7A1 and Ampho-HACOL7A1. Control, wild-type keratinocytes; NP185 mAb, anti-type VII collagen monoclonal antibody (orange, human specific); HA mAb, anti-human influenza hemagglutinin monoclonal antibody (orange); Hoechst 33342, nuclei (blue). Scale bar, 100 μ m. **(C)** Recessive dystrophic epidermolysis bullosa (RDEB) keratinocytes infected with GALV-COL7A1 and GALV-HACOL7A1. FNC1 pAb, anti-type VII collagen polyclonal antibody (orange); Control, type VII collagen-negative RDEB keratinocytes; note lack of type VII collagen in RDEB cells with FNC1 or HA antibodies. Scale bar, 100 μ m. **(D)** Quantification of virus transduction efficiency, using Ampho- and GALV-pseudotyped viral supernatants from **(B)** and **(C)**. **(E)** Western blot analysis of type VII collagen and HA epitope-tagged type VII collagen expression in wild-type and RDEB keratinocytes. Note that RDEB keratinocytes are null for type VII collagen; KC (w.t.), wild-type keratinocytes; FNC1 pAb, anti-type VII collagen polyclonal antibody; WB, Western blot. Protein molecular weight markers are displayed on the left. Arrows indicate the band corresponding to type VII collagen.

HACOL7A1 (N-terminal HA tagged) were produced in Phoenix packaging cells and tested for type VII collagen expression after infection into wild-type keratinocytes (Fig. 1B and D). Compared with control cells, transduced cells displayed clearly visible exogenous type VII collagen, using the human species-specific monoclonal antibody NP185 (Fig. 1B). Moreover, HA epitope-tagged type VII collagen expression was detected with both monoclonal anti-HA epitope (HA mAb; Fig. 1B) and anti-type VII collagen (NP185 mAb; Fig. 1B) antibody, confirming expression of the epitope-tagged procollagen protein in keratinocytes.

The large cDNA size of *COL7A1* has a negative impact on virus packaging, causing a reduction in viral titer. Also, generation of a single cell-derived packaging clone desirable for clinical-grade virus production is difficult regarding amphotropic virus and Phoenix cells because of the potential for self-infection of the producer cells. Therefore, we decided to pseudotype both viruses with the gibbon ape leukemia virus (GALV) envelope protein and establish single-cell packaging clones derived from the xenotropic packaging cell line PG13 (Miller *et al.*, 1991). The GALV-pseudotyped virus is not efficient in self-infecting PG13 packaging cells, reducing the possibility of a recombination effect in the parental packaging line. Ampho-COL7A1 and ampho-HACOL7A1 viruses were used to infect the PG13 packaging cell line and a virus-producing population of packaging clones derived from a single cell was established. The supernatants from such clones were screened by RT-PCR techniques (see Supplementary Fig. 1A at www.liebertonline.com/hum). More than 400 PG13 colonies were analyzed in order to establish high-efficiency virus-producing packaging clones. Colonies positive for COL7A1 by RT-PCR were subsequently screened for efficient infection of RDEB keratinocytes. PG13/HACOL7A1 xenotropic virus-packaging clones were identified by a similar approach.

RT-PCR-positive PG13 clones displayed variable titers, as measured by infection of type VII collagen-negative RDEB keratinocytes (see Supplementary Fig. 1B and C at www.liebertonline.com/hum). The highest viral titer-producing PG13 clones, 4D7 and F8, were further characterized for protein expression via immunofluorescence and Western blot analysis (Fig. 1C and E). After transduction, the secreted form of full-length type VII collagen was detected in both RDEB and wild-type keratinocytes. The epitope-tagged type VII collagen was detectable with either anti-type VII collagen FNC1 polyclonal antibody or antibodies directed toward the HA tag. This result corroborated the immunofluorescence data (Fig. 1B and C), indicating that the HA tag has no influence on type VII collagen protein expression. Furthermore, despite the negative impacts of large inserts on MLV-based viral titers, GALV pseudotyping in conjunction with large-scale screening allowed us to establish PG13/LZRSE-COL7A1 virus-producing clones 4D7 and F8 with transduction efficiencies as high as 93.2% (Fig. 1C and D) for clone 4D7 at an MOI equal to 15 in RDEB keratinocytes. The viral supernatant derived from these clones was further used to infect keratinocytes designated for *in vivo* grafting experiments.

Durability of type VII collagen expression *in vivo*

Assessment of the durability of gene expression in human skin tissue regenerated on immune-deficient mice is often hindered by the short life span of human skin grafts on im-

munocompromised animals. To evaluate the durability of type VII collagen expression *in vivo*, we used the HA-tagged retroviral construct expressed from the same LTR promoter as the wild-type gene. The transplantable human skin graft was generated by combining wild-type primary human keratinocytes infected with HACOL7A1 virus with human dermis. To track genetically modified epidermal cells non-invasively *in vivo*, keratinocytes were also infected with a luciferase reporter virus. Bioluminescence measurements were taken for 12 months postgrafting (Fig. 2A and B). The maximal bioluminescence signal was detected immediately after transplantation (Fig. 2B), reaching 3.8×10^7 photons/sec, with a further decline in intensity to 1.3×10^7 photons/sec within the first 2 months of the experiment. After the human skin graft had fully stabilized, the bioluminescence signal reached equilibrium at 1.5×10^7 photons/sec and remained at this steady state level for the duration of the study (Fig. 2A and B). Note that the luminescence signal remained localized within the human skin graft throughout the entirety of the experiment, indicating that the genetically modified keratinocytes did not migrate out from the transplanted skin. Thus, the LTR promoter provided stable, high-efficiency reporter gene expression in human skin with a duration of at least 12 months after skin transplantation.

Consistent with sustained bioluminescence, type VII collagen expression within the same grafted tissue persisted at each time point starting from 2 months through 12 months posttransplantation (Fig. 3A and B), with continuous proviral genome retained throughout the time course experiment (see Supplementary Fig. 4C at www.liebertonline.com/hum). In agreement with a lack of 100% transduction in our starting population, epitope-tagged type VII collagen was detectable within regions of the epidermis in 35 to 60% of epidermal cells between skip areas that lacked expression. Consistent with the ubiquitous expression of the LTR promoter, type VII collagen protein could be detected throughout all epidermal layers; however, no adverse effects of this were observed as indicated by normal tissue morphology (Fig. 3A) and the absence of elevated apoptosis (see Supplementary Fig. 4A and B at www.liebertonline.com/hum) in grafted skin. The human origin of the epidermis was confirmed via anti-desmoglein-3 monoclonal antibody staining (Fig. 3A). As shown in control panels, anti-desmoglein-3 antibody interacts only with human desmoglein-3 protein, resulting in a characteristic epidermal staining pattern, which is absent in mouse skin (Fig. 3A). Moreover, expression of the epidermal markers keratin-14, keratin-10, and loricrin in grafted skin (Fig. 3B) was comparable to that in normal human skin (Fig. 3B; and see Supplementary Fig. 2 at www.liebertonline.com/hum), indicating that HA-type VII collagen failed to disrupt epidermal morphology and differentiation in regenerated skin tissue *in vivo*.

To confirm that epitope-tagged type VII collagen assembled correctly at the BMZ, immunoelectron microscopy (immuno-EM) analysis of HACOL7A1-regenerated skin grafts was performed with anti-HA monoclonal antibody (Fig. 3C). HA-recombinant antigen demonstrated specific association with anchoring fibrils (black dots; Fig. 3C) at the BMZ with an efficiency level of at least $70 \pm 5\%$, confirming the immunofluorescence results and indicating that the epitope-tagged type VII collagen localizes within the same ultrastructural entities as does the wild-type protein.

FIG. 2. Durability of the LTR promoter in human epidermis. **(A)** Time course of bioluminescence in HACOL7A1-Luciferase transgenic human skin grafts representative of each time point. **(B)** Bioluminescent signal time course, quantitated as photons per second and graphed ($n=3$; mean $\pm$ SD shown).

Correction of the RDEB phenotype

To restore type VII collagen expression in RDEB tissue *in vivo* by this gene delivery approach, an epidermal grafting model was used, consisting of skin equivalents derived from GALV-COL7A1 virus-transduced RDEB patient keratinocytes and porcine dermis. The grafts were observed for up to 8 weeks posttransplantation and type VII collagen expression, protein localization, and skin morphology, including epidermal marker analysis, were evaluated *in vivo*.

In contrast to uncorrected RDEB skin grafts, epidermis regenerated with COL7A-transduced RDEB cells displayed

human type VII collagen at the BMZ (Fig. 4a and b). The anti-human type VII collagen antibody, NP185, used for these studies was highly species specific and did not cross-react with porcine or mouse type VII collagen (see Supplementary Fig. 3 at www.liebertonline.com/hum). Thus, the protein detected only in corrected RDEB skin grafts (Fig. 4a) was a consequence of LTR-mediated expression of exogenous type VII collagen and not an artifact of the nonspecific interaction with epitopes derived from other species, such as mouse or pig. Anti-desmoglein-3 staining of serial tissue sections was also performed to validate the human origin of the keratinocytes comprising the graft (Fig. 4b; and see Supplementary

FIG. 3. Sustainability of type VII collagen expression in human skin. **(A)** HA-tagged type VII collagen expression in human skin grafts over 1 year. HACOL7A1, HA epitope-tagged type VII collagen (linear orange staining at basement membrane zone [BMZ]); HA pAb, anti-human influenza hemagglutinin polyclonal antibody (orange); Desmoglein-3, anti-human desmoglein-3 mAb (marker for human origin of tissue; green); Hoechst 33342, nuclei (blue). Scale bars, 100 μ m ($n = 22$ mice). **(B)** Differentiation marker expression in human skin grafts 6 months posttransplantation. HA mAb, anti-human influenza hemagglutinin rat monoclonal antibody (orange). Note orange linear staining at the BMZ. Epidermal markers: keratin-14 (anti-K14 pAb, green), keratin-10 (anti-K10 pAb, green), and loricrin (anti-loricrin pAb, green). Scale bar, 100 μ m. **(C)** Immuno-EM analysis of HA-tagged type VII collagen in epidermal skin grafts regenerated with HACOL7A1-transduced keratinocytes 6 months postgrafting. Tissue sections were labeled *en bloc* with a human-specific anti-HA monoclonal antibody (HA mAb), followed by anti-mouse IgG-conjugated immunogold particles (black dots); AF, anchoring fibrils; LD, lamina densa; E, epidermis; D, dermis. Scale bar, 200 nm.

FIG. 4. Restoration of type VII collagen expression in RDEB human epidermis 8 weeks posttransplantation. **(a)** Type VII collagen expression. RDEB graft, regenerated from type VII collagen-negative RDEB keratinocytes and porcine dermis ($n = 3$ mice); Control skin graft, regenerated from normal (wild-type) keratinocytes and porcine dermis ($n = 3$ mice); NP185 mAb, anti-type VII collagen mAb (human specific, orange); Hoechst 33342, nuclei (blue). Note linear orange staining of type VII collagen at the BMZ of corrected and normal control tissue grafts. Scale bar, $100\ \mu\text{m}$. **(b)** Epidermal marker analysis in corrected skin. FNC1 pAb, anti-type VII collagen polyclonal antibody (orange); Desmoglein-3 mAb, anti-human desmoglein-3 (marker for human origin of tissue; green); K14 pAb, anti-keratin-14 polyclonal antibody (green); K10 pAb, anti-keratin-10 polyclonal antibody (green); and loricrin pAb (anti-loricrin polyclonal antibody, green). Scale bar, $100\ \mu\text{m}$ ($n = 3$ mice). **(c)** Histological analysis of the RDEB skin grafts 4 and 8 weeks posttransplantation. Normal control graft consists of pig dermis and epidermis prepared from wild-type keratinocytes. Control skin derived from human, mouse, and pig is indicated in the bottom row. **(d)** Immuno-EM analysis of a representative corrected RDEB skin sample 8 weeks postgrafting. Tissue sections were labeled *en bloc* with a human-specific anti-type VII collagen monoclonal antibody, NP185, followed by anti-mouse IgG-conjugated immunogold particles (black dots). AF, anchoring fibrils; LD, lamina densa; HD, hemidesmosome; E, epidermis; D, dermis. Scale bar, $100\ \text{nm}$.

Fig. 3 at www.liebertonline.com/hum). Expression of the epidermal markers keratin-14, keratin-10, and loricrin correlated well with the wild-type human skin, confirming that type VII collagen reexpression did not disrupt epidermal morphology and differentiation in RDEB skin tissue regenerated *in vivo* (Fig. 4b and c). Immuno-EM verified the correct localization of delivered type VII collagen in $92 \pm 5\%$ of anchoring fibrils (black dots, Fig. 4d), confirming IF results and suggesting that exogenously expressed type VII collagen from the retroviral LZRSE-COL7A1 vector is functionally indistinguishable from the endogenous wild-type protein and therefore capable of correcting the RDEB phenotype.

Discussion

Current treatment for RDEB is restricted to palliative wound care, underscoring the importance of developing clinically applicable therapeutics for this devastating disease. Preclinical studies have demonstrated significant progress for a variety of therapeutic approaches, showing successful restoration of type VII collagen expression at the dermal-epidermal junction. These studies have used direct gene (Woodley *et al.*, 2004b) and protein (Woodley *et al.*, 2004a; Remington *et al.*, 2009) transfer as well as cell-based therapies using epidermal keratinocytes (Chen *et al.*, 2002; Ortiz-Urda *et al.*, 2002; Gache *et al.*, 2004), dermal fibroblasts (Ortiz-Urda *et al.*, 2003; Woodley *et al.*, 2003; Kern *et al.*, 2009), or even stem cells from bone marrow transplantation (Tolar *et al.*, 2009). Although fibroblast- and protein-based therapies display attractive features, the short half-life of fibroblasts delivered to the dermis, as well as challenges in incorporating normal human type VII collagen into BMZ in clinical settings (Wong *et al.*, 2008), supports the search for improved methods of administration in patients. At present, only *ex vivo* retroviral delivery to autologous keratinocytes followed by skin grafting has thus far led to successful correction of the JEB subtype in the clinical setting (Mavilio *et al.*, 2006). As a main source of type VII collagen-producing cells *in vivo* (Burgeson, 1993), autologous cultured keratinocytes have been widely used for the permanent coverage of severe full-thickness burns with further indication of the long-term reepithelialization of the grafted sites (De Luca *et al.*, 1989; Pellegrini *et al.*, 1998). Moreover, earlier preclinical studies showed successful correction of the RDEB phenotype using *ex vivo* gene therapy and keratinocytes modified with either viral (Chen *et al.*, 2002; Gache *et al.*, 2004) or nonviral (Ortiz-Urda *et al.*, 2002) vectors. These studies suggested that an *ex vivo* gene therapy approach for RDEB, using autologous primary keratinocytes and retroviral vectors, will be both clinically applicable and feasible; however, the ability of this approach to sustain gene delivery for clinically meaningful durations had not been addressed.

Normal human epidermis undergoes continuous self-renewal orchestrated by progenitor cells resident in the basal layer of the skin; thus, any efficacious gene therapy for RDEB must efficiently target these progenitors. Despite the biosafety concerns discussed below, viruses remain one of the most efficient mechanisms for the introduction of genes in primary cells. Therefore, we constructed a retroviral vector, LZRSE-COL71, and investigated type VII collagen expression in primary keratinocytes *in vivo*. Retroviral titers are inversely correlated with the size of the insert. Optimization

of the envelope protein as well as thorough screening of the PG13 clones allowed us not only to establish a system for clinical-grade virus production, but also to achieve close to 90% transduction efficiency *in vitro* despite the exceptionally large size of the COL7A1 gene. This rate of gene transfer proved sufficient to target enough keratinocyte progenitors to obtain self-renewing epithelia for >12 epidermal turnover cycles *in vivo*.

In addition to efficient gene transfer to progenitors, long-term genetic correction depends on stable gene expression; therefore, the epigenetic silencing of LTR-driven transcription over time represents another potential concern. To address the durability of our vector in fully human skin grafts, we characterized the long-term expression of both an epitope-tagged type VII collagen that otherwise fully resembled the target recombinant vector designated for clinical use, as well as the luciferase marker gene. Both HA and luciferase were clearly detectable for more than 1 year postgrafting, indicating that silencing of these vectors does not occur in this time frame. Importantly, exogenous protein expression was observed within the human skin grafts, suggesting that epidermal progenitors had been effectively targeted. Moreover, type VII collagen expression was properly localized to anchoring fibrils of the BMZ, as confirmed via EM analysis (Fig. 3C). Although the relatively long half-life of type VII collagen in skin can permit protein detection after many epidermal renewing cycles, the fact that luciferase, which is relatively short-lived (Thompson *et al.*, 1991), can be detected at constant levels within the same graft even after 1 year of transduction argues that the MoMLV LTR promoter is active for the entire life span of the human skin transplant. Considering that human epidermis is renewed constantly and replaced monthly during life (Green, 1980), our finding translates into LTR durability for at least 12 epidermal turn-around cycles. Thus, the LZRS retroviral backbone is capable of both efficiently delivering COL7A1 to primary keratinocytes and driving gene expression for extended periods of time *in vivo*, suggesting that the LZRSE-COL7A1 vector may have clinical utility for the treatment of RDEB.

Another major challenge for retrovirus-based therapeutics is biosafety. The insertional activation of the LMO2 proto-oncogene and subsequent induction of leukemia in patients undergoing a hematopoietic stem cell human gene therapy trial (Hacein-Bey-Abina *et al.*, 2003a,b) precipitated a careful investigation of the use of retroviruses in human trials. The board of directors of the American Society of Gene Therapy (ASGT) has provided a broad survey of the comprehensive data on preclinical and clinical studies using retroviral vectors and reported that insertional gene activation in mice was estimated as 1 in 4846. Furthermore, in large animal studies no animals exposed to helper-free vector preparations developed retrovirus-mediated insertional oncogenesis (Kohn *et al.*, 2003). Likewise, we have never observed a spontaneous carcinoma in all our experience with regenerated human skin grafts prepared from retrovirally transduced human keratinocytes (Choate *et al.*, 1996a,b; Deng *et al.*, 1997, 1998; Seitz *et al.*, 1999; Robbins *et al.*, 2001). In fact, examination of the histology, as well as expression patterns, of skin epidermal markers keratin-14, keratin-10, and loricrin in both corrected RDEB and transduced wild-type skin transplants showed correlation with normal skin without any detectable abnormalities (Figs. 3 and 4). Moreover, careful analysis of the

bioluminescence images (Fig. 2A) demonstrates a localized signal detectable only within the human skin transplant. Detection of luciferase-labeled cells *in vivo* is extremely sensitive and can distinguish signals over background from as few as 1000 human cells distributed throughout the peritoneal cavity of a mouse, with linear relationships between cell number and signal intensity over 5 logs (Edinger *et al.*, 1999). Localization of the bioluminescent signal exclusively within grafted skin argues for the inability of either the retrovirus or transduced keratinocytes to migrate outside of the grafted area even 12 months after skin transplantation. In light of the LMO2-induced leukemia, the biosafety of retroviruses will always remain a concern; however, the accessibility of skin to rapid and direct excisional surgery provides an additional safeguard should malignancies arise after gene transfer in this setting.

Type VII expression in RDEB may potentially trigger an unwanted immune reaction in patients lacking all type VII protein, highlighting the need for care in selecting initial subjects for trials of the approach reported here. In this regard, the amino-terminal noncollagenous domain of type VII collagen is a target of autoantibodies in the acquired immune disorder epidermolysis bullosa acquisita (EBA) (Lapiere *et al.*, 1993). Although the actual mechanism of autoantibody production in EBA is unknown, it is clear from clinical studies of EBA that type VII collagen antibodies are capable of inducing significant skin pathology consisting of sublamina densa separation of skin and mucosa and subsequent dystrophic scarring. Moreover, a protein therapy approach using recombinant type VII collagen injected into *Col7a1* knockout mice induced elevated titers of anti-collagen VII antibodies, although these antibodies were absent at the BMZ and did not interfere with newly delivered protein incorporation into mouse skin (Remington *et al.*, 2009). The 60% or more of patients with RDEB who retain expression of truncated NC1-containing type VII collagen protein, the most antigenic region of the protein (Ortiz-Urda *et al.*, 2005), may thus represent the most attractive initial population for assessment of this approach.

Our findings indicate that retroviral gene transfer using the LZRS-COL7A1 vector may support durable corrective gene delivery in RDEB skin tissue *in vivo*. Correction of the RDEB phenotype was observed at the levels of cell and tissue morphology and anchoring fibril formation. The presented results thus demonstrate sustainable RDEB phenotypic correction as well as underscore the therapeutic potential of this approach being pursued for genetic correction in patients with RDEB. As such, these preclinical studies have enabled approval of a human phase I clinical trial of *ex vivo* gene delivery in RDEB by the U.S. Food and Drug Administration (BB-IND-13708).

Acknowledgments

The authors thank Jason Reuter for important contributions to discussions throughout this work; and Florence Scholl, Todd Ridky, and Carolyn Lee for technical advice, support, and helpful discussions. The authors are also grateful to Douglas Keene for electron microscopy analysis. This work was supported by the U.S. Veterans Affairs Office of Research and Development, by NIH grant AR055914, and by the Epidermolysis Bullosa Medical Research Foundation.

Author Disclosure Statement

The authors declare no competing financial interest.

References

- Burgeson, R.E. (1993). Type VII collagen, anchoring fibrils, and epidermolysis bullosa. *J. Invest. Dermatol.* 101, 252–255.
- Chen, M., Kasahara, N., Keene, D.R., Chan, L., Hoeffler, W.K., Finlay, D., Barcova, M., Cannon, P.M., Mazurek, C., and Woodley, D.T. (2002). Restoration of type VII collagen expression and function in dystrophic epidermolysis bullosa. *Nat. Genet.* 32, 670–675.
- Choate, K.A., Kinsella, T.M., Williams, M.L., Nolan, G.P., and Khavari, P.A. (1996a). Transglutaminase 1 delivery to lamellar ichthyosis keratinocytes. *Hum. Gene Ther.* 7, 2247–2253.
- Choate, K.A., Medalie, D.A., Morgan, J.R., and Khavari, P.A. (1996b). Corrective gene transfer in the human skin disorder lamellar ichthyosis. *Nat. Med.* 2, 1263–1267.
- Christiano, A.M., Greenspan, D.S., Lees, and Uitto, J. (1994a). Cloning of human type VII collagen. Complete primary sequence of the α_1 (VII) chain and identification of intragenic polymorphisms. *J. Biol. Chem.* 269, 20256–20262.
- Christiano, A.M., Hoffman, G.G., Chung-Honet, L.C., Lee, S., Cheng, W., Uitto, J., and Greenspan, D.S. (1994b). Structural organization of the human type VII collagen gene (*COL7A1*), composed of more exons than any previously characterized gene. *Genomics* 21, 169–179.
- De Luca, M., Albanese, E., Bondanza, S., Megna, M., Ugozzoli, L., Molina, F., Cancedda, R., Santi, P.L., Bormioli, M., Stella, M., and Magliacani, G. (1989). Multicentre experience in the treatment of burns with autologous and allogenic cultured epithelium, fresh or preserved in a frozen state. *Burns* 15, 303–309.
- Deng, H., Lin, Q., and Khavari, P.A. (1997). Sustainable cutaneous gene delivery. *Nat. Biotechnol.* 15, 1388–1391.
- Deng, H., Choate, K.A., Un, Q., and Khavari, P.A. (1998). High-efficiency gene transfer and pharmacologic selection of genetically engineered human keratinocytes. *Biotechniques* 25, 274–280.
- Di Nunzio, F., Maruggi, G., Ferrari, S., Di Iorio, E., Poletti, V., Garcia, M., Del Rio, M., De Luca, M., Larcher, F., Pellegrini, G., and Mavilio, F. (2008). Correction of laminin-5 deficiency in human epidermal stem cells by transcriptionally targeted lentiviral vectors. *Mol. Ther.* 16, 1977–1985.
- Eady, R.A., and Dunnill, M.G. (1994). Epidermolysis bullosa: Hereditary skin fragility diseases as paradigms in cell biology. *Arch. Dermatol. Res.* 287, 2–9.
- Edinger, M., Svveaney, T.J., Tucker, A.A., Olomu, A.B., Negrin, R.S., and Contag, C.H. (1999). Noninvasive assessment of tumor cell proliferation in animal models. *Neoplasia* 1, 303–310.
- Gache, Y., Baldeschi, C., Del Rio, M., Gagnoux-Palacios, L., Larcher, F., Lacour, J.P., and Meneguzzi, G. (2004). Construction of skin equivalents for gene therapy of recessive dystrophic epidermolysis bullosa. *Hum. Gene Ther.* 15, 921–933.
- Green, H. (1980). The keratinocyte as differentiated cell type. *Harvey Lect.* 74, 101–139.
- Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., Le Deist, F., Wulffraat, N., McIntyre, E., Radford, I., Villeval, J.L., Fraser, C.C., Cavazzana-Calvo, M., and Fischer, A. (2003a). A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* 348, 255–256.
- Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M.P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C.S.,

- Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., Fraser, P., Cohen, J.I., de Saint Basile, G., Alexander, I., Wintergerst, U., Frebourg, T., Aurias, A., Stoppa-Lyonnet, D., Romana, S., Radford-Weiss, I., Gross, F., Valensi, F., Delabesse, E., Macintyre, E., Sigaux, F., Soulier, J., Leiva, L.E., Wissler, M., Prinz, C., Rabbitts, T.H., Le Deist, F., Fischer, A., and Cavazzana-Calvo, M. (2003b). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 302, 415–419.
- Heagerty, A.H., Kennedy, A.R., Leigh, I.M., Purkis, P., and Eady, R.A. (1986). Identification of an epidermal basement membrane defect in recessive forms of dystrophic epidermolysis bullosa by LH 7:2 monoclonal antibody: Use in diagnosis. *Br. J. Dermatol.* 115, 125–131.
- Kern, J.S., Loeckermann, S., Fritsch, A., Hausser, L., Roth, W., Magin, T.M., Mack, C., Muller, M.L., Paul, O., Ruther, P., and Bruckner-Tuderman, L. (2009). Mechanisms of fibroblast cell therapy for dystrophic epidermolysis bullosa: High stability of collagen VII favors long-term skin integrity. *Mol. Ther.* 17, 1605–1615.
- Kinsella, T.M., and Nolan, G.P. (1996). Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum. Gene Ther.* 7, 1405–1413.
- Kohn, D.B., Sadelain, M., Dunbar, C., Bodine, D., Kiem, H.P., Candotti, F., Tisdale, J., Riviere, I., Blau, C.A., Richard, R.E., Sorrentino, B., Nolte, J., Malech, H., Brenner, M., Cornetta, K., Cavagnaro, J., High, K., Glorioso, J.; American Society of Gene Therapy (ASGT). (2003). American Society of Gene Therapy (ASGT) ad hoc subcommittee on retroviral-mediated gene transfer to hematopoietic stem cells. *Mol. Ther.* 8, 180–187.
- Korge, B.P., and Krieg, T. (1996). The molecular basis for inherited bullous diseases. *J. Mol. Med.* 74, 59–70.
- Lapierre, J.C., Woodley, D.T., Parente, M.G., Iwasaki, T., Wynn, K.C., Christiano, A.M., and Uitto, J. (1993). Epitope mapping of type VII collagen: Identification of discrete peptide sequences recognized by sera from patients with acquired epidermolysis bullosa. *J. Clin. Invest.* 92, 1831–1839.
- Leigh, I.M., Eady, R.A., Heagerty, A.H., Purkis, P.E., Whitehead, P.A., and Burgeson, R.E. (1988). Type VII collagen is a normal component of epidermal basement membrane, which shows altered expression in recessive dystrophic epidermolysis bullosa. *J. Invest. Dermatol.* 90, 639–642.
- Marinkovich, M.P. (1993). The molecular genetics of basement membrane diseases. *Arch. Dermatol.* 129, 1557–1565.
- Mavilio, F., Pellegrini, G., Ferrari, S., Di Nunzio, F., Di Iorio, E., Recchia, A., Maruggi, G., Ferrari, G., Provasi, E., Bonini, C., Capurro, S., Conti, A., Magnoni, C., Giannetti, A., and De Luca, M. (2006). Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells. *Nat. Med.* 12, 1397–1402.
- Miller, A.D., Garcia, J.V., Von Suhr, N., Lynch, C.M., Wilson, C., and Eiden, M.V. (1991). Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. *J. Virol.* 65, 2220–2224.
- Ortiz-Urda, S., Thyagarajan, B., Keene, D.R., Un, Q., Fang, M., Calos, M.P., and Khavari, P.A. (2002). Stable nonviral genetic correction of inherited human skin disease. *Nat. Med.* 8, 1166–1170.
- Ortiz-Urda, S., Lin, Q., Green, C.L., Keene, D.R., Marinkovich, M.P., and Khavari, P.A. (2003). Injection of genetically engineered fibroblasts corrects regenerated human epidermolysis bullosa skin tissue. *J. Clin. Invest.* 111, 251–255.
- Ortiz-Urda, S., Garcia, J., Green, C.L., Chen, L., Un, Q., Veitch, D.P., Sakai, L.Y., Lee, H., Marinkovich, M.P., and Khavari, P.A. (2005). Type VII collagen is required for Ras-driven human epidermal tumorigenesis. *Science* 307, 1773–1776.
- Paller, A.S. (1996). The genetic basis of hereditary blistering disorders. *Curr. Opin. Pediatr.* 8, 367–371.
- Pellegrini, G., Bondanza, S., Guerra, L., and De Luca, M. (1998). Cultivation of human keratinocyte stem cells: Current and future clinical applications. *Med. Biol. Eng. Comput.* 36, 778–790.
- Remington, J., Wang, X., Hou, Y., Zhou, H., Burnett, J., Muirhead, T., Uitto, J., Keene, D.R., Woodley, D.T., and Chen, M. (2009). Injection of recombinant human type VII collagen corrects the disease phenotype in a murine model of dystrophic epidermolysis bullosa. *Mol. Ther.* 17, 26–33.
- Robbins, P.B., Lin, Q., Goodnough, J.B., Tian, F.L., Chen, X., and Khavari, P.A. (2001). *In vivo* restoration of laminin 5 $\beta 3$ expression and function in junctional epidermolysis bullosa. *Proc. Natl. Acad. Sci. U.S.A.* 98, 5193–5198.
- Seitz, C.S., Giudice, G.J., Balding, S.D., Marinkovich, M.P., and Khavari, P.A. (1999). BP180 gene delivery in junctional epidermolysis bullosa. *Gene Ther.* 6, 42–47.
- Thompson, J.F., Hayes, L.S., and Lloyd, D.B. (1991). Modulation of firefly luciferase stability and impact on studies of gene regulation. *Gene* 103, 171–177.
- Tolar, J., Ishida-Yamamoto, A., Riddle, M., McElmurry, R.T., Osborn, M., Xia, L., Lund, T., Slattery, C., Ditto, J., Christiano, A.M., Wagner, J.E., and Blazar, B.R. (2009). Amelioration of epidermolysis bullosa by transfer of wild-type bone marrow cells. *Blood* 113, 1167–1174.
- Uitto, J., and Pulkkinen, L. (1996). Molecular complexity of the cutaneous basement membrane zone. *Mol. Biol. Rep.* 23, 35–46.
- Wong, T., Gammon, L., Liu, L., Mellerio, J.E., Dopping-Hepenstal, P.J., Pacy, J., Elia, G., Jeffery, R., Leigh, I.M., Navsaria, H., and McGrath, J.A. (2008). Potential of fibroblast cell therapy for recessive dystrophic epidermolysis bullosa. *J. Invest. Dermatol.* 128, 2179–2189.
- Woodley, D.T., Krueger, G.G., Jorgensen, C.M., Fairley, J.A., Atha, T., Huang, Y., Cliau, L., Keene, D.R., and Chen, M. (2003). Normal and gene-corrected dystrophic epidermolysis bullosa fibroblasts alone can produce type VII collagen at the basement membrane zone. *J. Invest. Dermatol.* 121, 1021–1028.
- Woodley, D.T., Keene, D.R., Atha, T., Huang, Y., Lipman, K., Li, W., and Chen, M. (2004a). Injection of recombinant human type VII collagen restores collagen function in dystrophic epidermolysis bullosa. *Nat. Med.* 10, 693–695.
- Woodley, D.T., Keene, D.R., Atha, T., Huang, Y., Ram, R., Kasahara, N., and Chen, M. (2004b). Intradermal injection of lentiviral vectors corrects regenerated human dystrophic epidermolysis bullosa skin tissue *in vivo*. *Mol. Ther.* 10, 318–326.

Address correspondence to:

Dr. Paul A. Khavari

Program in Epithelial Biology

269 Campus Drive, Room 2145

Stanford University School of Medicine

Stanford, CA 94305

E-mail: khavari@stanford.edu

Received for publication February 6, 2010;

accepted after revision May 24, 2010.

Published online: September 16, 2010.