Gene Therapy (2003) 10, 1099–1104
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www.nature.com/gt

RESEARCH ARTICLE

Sustainable correction of junctional epidermolysis bullosa via transposon-mediated nonviral gene transfer

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Sustainable correction of severe human genetic disorders of self-renewing tissues, such as the blistering skin disease junctional epidermolysis bullosa (JEB), is facilitated by stable genomic integration of therapeutic genes into somatic tissue stem cells. While integrating viral vectors can achieve this, they suffer from logistical and biosafety concerns. To circumvent these limitations, we used the Sleeping Beauty *transposable element to integrate the LAMB3 cDNA into genomes of epidermal holoclones from six unrelated JEB patients. These cells regenerate human JEB skin that is normalized at the level of laminin 5 protein expression, hemidesmosome formation and blistering. Transposon-mediated gene delivery therefore affords an opportunity for stable gene delivery in JEB and other human diseases.*
Gene Therapy (2003) **10,** 1099–1104. doi:10.1038/sj.gt.3301978

Keywords: *skin; epidermolysis bullosa; gene therapy*

Introduction

Therapeutic gene transfer to self-renewing somatic tissues has relied on viral vectors. As a result of their ability to achieve stable genomic integration into tissue stem cells, retroviruses have served as a mainstay of such efforts. Retroviral vectors are capable of targeting somatic stem cells and achieving durable gene delivery through multiple turnover cycles in tissues that undergo continual regeneration such as epidermis.[1,2] Retroviral gene transfer, however, suffers from the production, cost, storage and biosafety issues common to all viral vectors. While plasmid vectors are free of many of these drawbacks, they have been limited by transient episomal expression insufficient to support durable phenotypic reversion in skin and other tissues.[3–5]

Limitations of current viral and nonviral approaches to stable somatic tissue gene transfer have stimulated the search for nonviral approaches to stably integrate therapeutic genes. Among such approaches is plasmid-based gene transfer mediated by the *Sleeping Beauty* (SB) synthetic transposable element.[6–8] SB is an ancestral fish member of the Tc1/mariner family.[9] SB transposition integrates incoming DNA into the genome using a 'cut-and-paste' mechanism requiring short inverted repeat (IR) sequences flanking the insert and the SB transposase protein.[10] SB transposase has been shown to facilitate gene integration into mammalian cell lines and murine liver sufficient to support long-term gene delivery.[6,7] In spite of these attractive features, transposon-mediated gene transfer has not yet been reported capable of achieving stable genetic correction of human tissue from patients with characterized genetic disorders.

Severe junctional epidermolysis bullosa (JEB) is a genodermatosis characterized clinically by widespread epithelial fragility and blistering that commonly leads to death in the first years of life.[11] Mutations in a number of genes can underly lethal JEB, however, the *LAMB3* gene that encodes the laminin β3 subunit of laminin 5 is the most commonly affected.[11,12] Laminin 5 is a heterotrimer comprised of laminin α3, β3 and γ2 chains and serves a major constituent of the cutaneous basement membrane zone. It interacts physically with α6β4 integrin on the basal surface of epidermal keratinocytes to promote hemidesmosome formation as well as with Type VII collagen in dermal anchoring fibrils to enhance basement membrane zone integrity.[13,14] In facilitating these processes, laminin 5 plays a nonredundant role in epidermal–dermal adhesion.[12,15] Recently, Moloney murine leukemia-based amphotropic retroviruses have been used to transduce successfully *LAMB3* mutant stem cells from JEB patient epidermis.[2] While efficient, durable and capable of normalizing laminin 5 protein expression and hallmark JEB disease features, this approach suffers from limitations that include biosafety concerns as well as the expense and regulatory hurdles of viral gene transfer. Extending such corrective gene delivery to self-renewing human tissues towards widespread clinical application will benefit from nonviral approaches to durable gene transfer.

Here we demonstrate stable restoration of laminin 5 protein expression to human epidermal tissue from a series of six unrelated JEB patients using the SB

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Received 8 August 2002; accepted 6 December 2002

transposon. Gene transfer integrated the *LAMB3* cDNA into genomes of epidermal stem cell holoclones *in vitro*. Following short-term enrichment for corrected cells in culture, laminin 5 expressing JEB keratinocytes were used to regenerate human skin on immune-deficient mice. JEB skin regenerated from these cells displayed correction of key JEB disease characteristics, including reexpression of laminin 5 protein, restored hemidesmosome formation and lack of epidermal–dermal blistering. These data demonstrate that transposition-based gene transfer offers a feasible approach to stable gene delivery to self-renewing human tissues in the treatment of genetic diseases of skin and other tissues.

Results

Gene delivery to primary keratinocytes using the SB transposase

Nonviral plasmid-based vectors are free of many of the disadvantages that constrain viral vectors; however, conventional plasmids exist as episomes and thus achieve only transient gene transfer because such extragenomic DNA is unstable. To achieve stable plasmid-based nonviral gene transfer in JEB, we used an approach to permanent gene integration based on the SB ancestral fish transposon. An SB IR-flanked laminin β3 expression plasmid was constructed in which gene expression is directed by the CMV IE promoter driving both laminin β3 expression and the blastocidin resistance gene (pLAMB3) as well as lacZ marker control (pLacZ) (Figure 1). The CMV promoter used supports durable gene transfer to human epidermis and is active throughout the epidermis.[1,2,16] SB transposition has been shown to act with varying efficacy depending on the target cell type,[6] thus we first tested its functionality in primary cells of human skin. Normal keratinocytes were transfected with pLacZ with and without CMV-driven expression vectors encoding SB and an inactive SB mutant (mSB).[7] At 2 days after transfection, equivalent proportions of cells in each group were X-gal(+) (42 ± 1.3, 41 ± 0.8 and 43 ± 2.3 for pLacZ alone, pLacZ/SB and pLacZ/mSB, respectively). After 2 weeks of growth without selection, cells were subjected to X-gal staining to examine plasmid retention. As recently reported, both corrected and uncorrected JEB keratinocytes display normal growth kinetics when plated on extracellular matrix.[17] After selection, cells cotransfected with pLacZ and wild-type SB expression plasmids displayed 55 ± 4.5-fold more X-gal(+) cells (Figure 1b, c) with a total of $17.2\pm$% of unselected cells X-gal(+). The percentage of X-gal(+) in cells receiving either no SB or inactive mutant SB was 0.3 and 0.4%, respectively. Thus, SB transposase enhances the durability of gene delivery to primary human keratinocytes in culture.

Restoration of laminin 5 protein expression in epidermal holoclones

We next wished to extend findings with marker gene delivery in normal keratinocytes to *LAMB3* gene transfer in JEB. Primary keratinocytes from six previously characterized unrelated JEB patients with *LAMB3* mutations and absent laminin 5 protein expression[2] were transfected with pLAMB3 and either SB or mSB expression plasmids. After 10 days of growth without selection following transfection, JEB cells receiving wild-type transposase and pLAMB3 plasmids (JEB(+)) displayed restored expression of full-length laminin β3 protein; expression was undetectable in control JEB cells (Figure 2a). To enrich the percentage of transfected cells in the bulk population, a parallel set of cells were exposed to short-term drug selection for 10 days following transfection. Consistent with prior observations,[16] blastocidin selection exerted no effects on keratinocyte morphology or subsequent proliferative ability. At this timepoint, >99% of cells in JEB(+) bulk populations demonstrated normalized cellular expression of laminin β3 protein by immunohistochemistry (Figure 2b). To examine if transposase gene delivery targets epidermal stem cells, keratinocytes enriched with short-term selection were plated at limiting dilution and holoclones[18] were isolated based on high proliferative capacity as previously described.[2,19] Plasmid rescue was performed for 10 independently isolated holoclones. Sequence analysis of recovered transposon integrants showed SB-mediated transposition into TA dinucleotides in multiple different chromosomes (Table 1). None of these integrations appeared to reside within known intragenic sequences. These findings indicate that transposon-mediated gene transfer stably integrates delivered

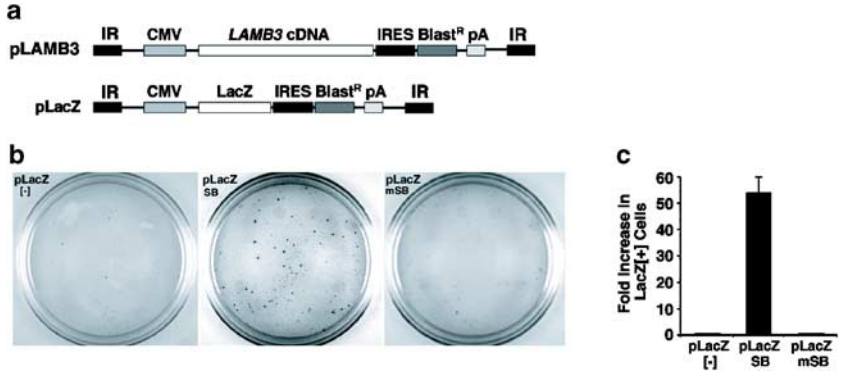

Figure 1 *Gene delivery to primary human keratinocytes using the SB transposase. (a) Design of transfer vectors for transposon-mediated gene delivery of laminin β3 (pLAMB3) and LacZ (pLacZ). (b) X-gal staining of unselected primary human epidermal cells after transfection with pLacZ in the absence (−) or presence of cotransfected wild-type (SB) or nonfunctional mutant (mSB) transposase expression plasmid followed by plating at low density and growth without selection. (c) Quantitation of stably expressing cells via optical computation scanning of triplicate independent transfections ±s.d.; pLacZ (−) is normalized to a value of 1.0.*

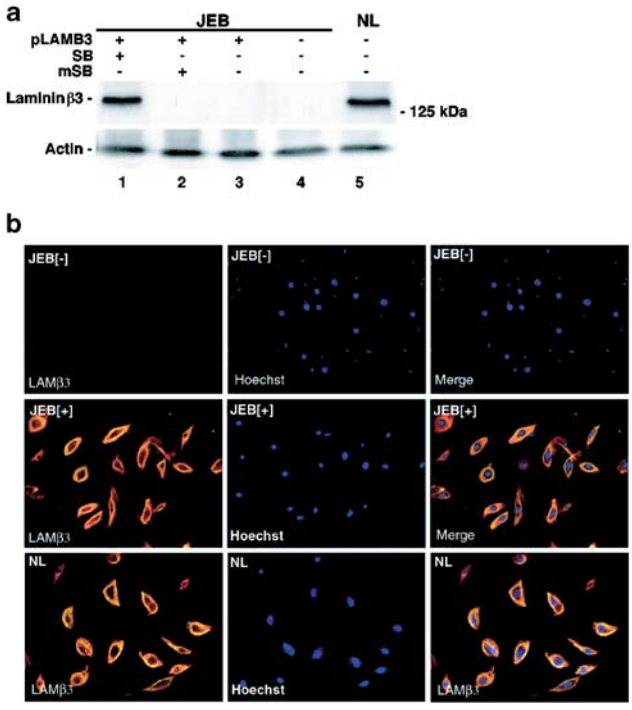

Figure 2 *Reexpression of full-length laminin 5 β3 protein expression to primary JEB keratinocytes. (a) Western blots of primary human epidermal cells JEB and normal control (NL) patients 14 days after transfection with (+) or without (−) pLAMB3 and wild-type (SB) and mutant (mSB) transposase expression plasmids and subsequent growth without selection. The top panel represents the 140 kDa full-length laminin 5 β3 protein and the bottom panel represents actin loading control. (b) Expression of laminin 5 β3 protein in untransfected (JEB(−), top row) and pLAMB3/SB transfected (JEB(+), middle row) primary JEB patient keratinocytes after 10 days of selection enrichment along with normal control (NL, bottom row). The left column represents immunostaining with antibody to laminin 5 β3 (LAMβ3), the middle panel Hoechst 33342 staining to highlight all cell nuclei and the right panels are merged images.*

gene sequences into the genomes of epidermal holoclones.

Laminin 5 gene delivery in JEB patient skin tissue in vivo

We next examined if transposon-based *LAMB3* gene transfer to epidermal holoclones corrected hallmark JEB disease features. Following 10 days selection enrichment in culture, pLAMB3-transfected cells were utilized to regenerate human skin on CB.17 *scid/scid* immunedeficient mice in an approach that accurately recapitulates the histologic, protein expression and functional features of the individual donor, including pathologic aspects of specific genetic skin diseases.[2,20–23] Similar to the donor skin on each of the series of JEB patients, skin regenerated from pLacZ transfected JEB skin demonstrated separation at the dermal–epidermal junction and no detectable laminin 5 expression was observed (Figure 3). In contrast, skin tissue regenerated from pLAMB3-transfected JEB cells from the same patients exhibited normal histology and an intact dermal–epidermal junction (Figure 3a). Moreover, laminin 5 was correctly distributed to the junction in a polarized manner indistinguishable from normal tissue (Figure 3b). While quantitating laminin 5 protein levels *in vivo* is difficult because of the insoluble nature of the protein, laminin 5 immunostaining intensity was similar to normal human controls, suggesting that protein restoration occurred in the physiologic range. In agreement with the observed targeting of holoclones, laminin 5 expression was stable, lasting for the 16-week duration of the experiment through multiple epidermal turnover cycles, defined previously in regenerated human skin as 3–4 weeks in length.[2] Thus, transposon-based gene transfer supports durable gene delivery and normalized laminin 5 protein expression to regenerated human JEB epidermis *in vivo*.

The ultrastructural consequences of laminin 5 absence in mice and humans include an absence of hemidesmosomes in the cutaneous basement membrane zone.[11,12] To examine if hemidesmosome formation was restored by expressed laminin 5 in regenerated JEB patient skin, we undertook immunoelectron microscopy. While uncorrected JEB skin lacked hemidesmosomes, these structures were restored in pLAMB3-engineered JEB skin (Figure 4). Immunogold labeling using species-specific antibodies to human laminin 5 labeled anchoring filaments extending inward from hemidesmosome structures and confirmed their human origin (Figure 4), confirming restoration of the major ultrastructural deficit in JEB.

Discussion

Our studies demonstrate that nonviral transposon-mediated gene delivery enables genetic correction of self-renewing human tissue from a life-threatening inherited skin disease. Prior approaches restored expression of a number of genes to epidermal cells in JEB and other skin diseases *in vitro*[24,25] and in regenerated tissue *in vivo*;[2,21–23] however, they relied on amphotropic retroviruses with all their attendant drawbacks. In the plasmid-based approach presented here, functional SB transposase is required for sustained corrective gene delivery because a nonfunctional mutant failed to maintain gene transfer, even *in vitro*. Stable gene delivery was achieved with integration of laminin β3 encoding sequences into genomes of holoclones of high proliferative capacity. Similar to prior viral studies with a promoter active in all epidermal layers, we found that the CMV promoter employed in the pLAMB3 plasmid sufficed to support correctly polarized laminin 5 protein expression. Thus, although vectors targeting epidermal regions using layer-specific promoters have been developed,[26] they have proven unnecessary for properly restored protein distribution in both the current study and in the other epidermal genodermatoses corrected *in vivo* to date.[2,21–23] This finding points to a post-transcriptional process, potentially facilitated by physical interactions with other laminin 5 chains and corresponding ligands, such as α6β4 integrin and Type VII collagen, that may direct correct laminin β3 distribution.[13,14] Normalization of JEB skin tissue occurred at the levels of tissue laminin 5 expression and hemidesmosome formation. The presence of hemidesmosomes, the restored expression of laminin 5 and the absence of junctional blistering are strong indicators that tissue cohesion was normalized. These findings suggest that transposase-mediated gene transfer will support efforts directed at stable genetic correction in JEB patients.

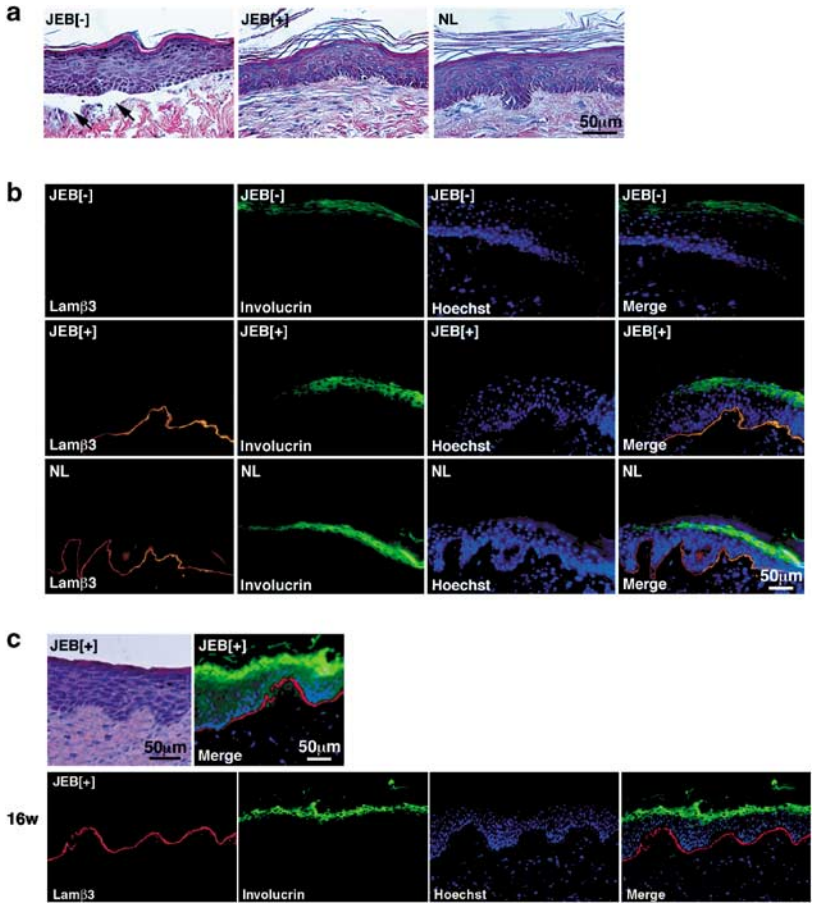

Figure 3 *Restoration of laminin 5 β3 protein expression in regenerated human JEB patient skin tissue in vivo. (a) Histologic appearance of human skin tissue regenerated on immune-deficient mice from primary patient keratinocytes transfected with LacZ control (JEB(−)), pLAMB3/SB (JEB(+)) as well as primary cells from normal patients (NL). Note areas of separation at the dermal–epidermal junction in JEB(−) tissue (arrows denote subepidermal blister) that are not seen in either normal or JEB(+) tissue. (b) Restoration of laminin 5 β3 protein expression in JEB skin. Expression of laminin 5 β3 protein in human skin 8 weeks after regeneration from primary patient keratinocytes transfected with LacZ control (JEB(−), top row), pLAMB3/SB (JEB(+), middle row) after short-course selection enrichment as well as primary cells from normal patients (NL, bottom row). Staining with human species-specific antibodies to laminin 5 β3 (first column of panels at left, orange) and the suprabasal layer involucrin differentiation marker (second column, green) are shown. Hoechst 33342 staining (third column, blue) is shown to highlight all cellular nuclei and the images in the last column merge the three preceding panels in the same row. Note restored expression of laminin 5 β3 protein to the correct basement membrane distribution in JEB(+) tissue in a fashion similar to normal. (c) Expression of laminin 5 β3 protein expression in JEB skin at 16 weeks postregeneration. Histology and merged immunohistochemical stain as in Figure 4b is shown.*

The approach used here relies upon a specific series of steps to achieve phenotypic correction of JEB skin. First, plasmids were introduced into primary JEB patient keratinocytes at >40% efficiency. Transfected cells were then enriched using a short course of drug selection, with integration driven by SB transposase coexpression then used to regenerate human skin. The fact that all studied holoclones of high proliferative capacity demonstrated SB-mediated transposition of laminin β3 encoding sequence confirmed that this corrected population contains the holoclone cells sufficient to sustain epidermal self-renewal.[2,27] Consistent with this, laminin 5 protein expression was maintained through multiple epidermal turnover cycles *in vivo*. For human epidermis, such self-renewal turnover cycles have been defined as of 3–4 weeks in duration.[2] This approach thus avoids pitfalls of prior plasmid-based efforts in keratinocytes where plasmid-driven expression is commonly lost by 3–5 days[3,5] and where long-term drug selection triggers differentiation with loss of proliferative capacity.[28]

Through expeditious production of genetically corrected holoclone-containing cell populations, the data described here afford a practical approach to nonviral gene therapy to self-renewing tissues in humans. Transposon-based plasmid integration thus provides the ability to achieve stable nonviral gene transfer through genomic integration in tissue stem cells and offers a rational alternative to current viral approaches.

Materials and methods

JEB patients and cell growth

Primary epidermal cells from laminin 5-deficient JEB patients meeting clinical, immunohistological, ultrastructural and genetic criteria for the disease were selected from the National Epidermolysis Bullosa Registry site at Stanford University.[2] Patients studied had confirmed *LAMB3* gene mutations and an absence of laminin 5 protein in skin, as defined by immunochemistry. Normal

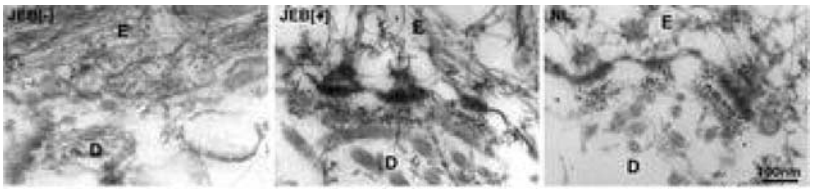

Figure 4 *Restoration of hemidesmosome formation to JEB skin tissue. Immunoelectron microscopy of human skin regenerated from primary JEB patient keratinocytes transfected with LacZ control (JEB(−)), pLAMB3/SB (JEB(+)) as well as primary cells from normal patients (NL). Note the absence of hemidesmosomes in uncorrected tissue and the restoration of these structures in JEB(+) tissue similar to normal control; black dots represent immunogold labeling of laminin 5 β3 protein in anchoring filaments using human species-specific antibodies to laminin 5 β3 (E=epidermal cell; D=dermis).*

Table 1 Genomic integrants in epidermal holoclones

Integration site sequences (IR … IR)

Clone			Integration Site	Homology
1	ctattgtggactgta**TA**	**TA**tctatgagcagagtt	5q35.1	1053/1090(97%)
2	agactctgctcctaa**TA**	**TA**gatcctgtggtatct	1p12	490/496 (98%)
3	tatgggctttagatg**TA**	**TA**attctcacaacagat	14q21.3	300/300 (100%)
4	attatataattgata**TA**	**TA**	1p31.1	479/483 (99%)
5	aatagagaccgtgct**TA**	**TA**gctcctgcttagacc	5p15.3	945/978 (97%)
6	gtctgcaagtggata**TA**	**TA**tggacctgtttgagg	7p22	472/492 (95%)
7	gtgtctcagcacaga**TA**	**TA**taacggaacattgtc	22q12.1	981/1000 (98%)
8	gctgtaaatgagaat**TA**	**TA**cctgtttaacatttt	17q25.1	675/695 (97%)
9	cctcagcctcctaag**TA**	**TA**gctaggactacaggt	19p13.3	242/243 (100%)
10	atatttaatgaaata**TA**	**TA**	5q14.3	289/290 (100%)

Plasmid rescue from 10 JEB(+) keratinocyte holoclones. Sequence, chromosomal location of integration site and degree of sequence homology to genomic sequence are shown.

control keratinocytes were obtained from unrelated individuals unaffected by EB. Cells were isolated and grown in culture in 50% serum-free medium (GIBCO/BRL) and 50% medium154 (Cascade Biologics) as described.[2] For holoclone characterization, cells were plated at limiting dilution and single holoclone colonies of high proliferative capacity[18] measuring >5 mm in diameter were isolated as described[2,19] and used for genomic integration studies.

Vectors and gene transfer

Plasmids: A *Bgl*II/*Drd*I fragment encoding LAMB3/LacZ IRES and Blastocidin resistance was cloned as a *Bgl*II/*Drd*I (blunt) fragment into the *Bgl*II/*Nhe*I (blunt) sites of pTMCS containing the inverted repeats 5IR and 3IR, producing the vector pTMCS LAMB3 (pLAMB3) and pTMCS LacZ (pLacZ). A plasmid containing a neomycin-phosphotransferase (neo) and kanamycin expression cassette flanked by SB inverted repeats (pTnori) was used for transposon rescue from keratinocyte genomes. Cells were transfected with pLAMB3 or with pLacZ and CMV-driven expression vectors[7] for either with wild-type transposase (pCMV-SB; SB) or inactive mutant transposase (pCMV-mSB; mSB) using modified polybrene shock as described.[29] Cells were either grown without selection for assessment of sustained gene expression or subjected to 10 days of blasticidin selection (4μg/ml). Gene transfer efficiency was verified by immunofluorescence microscopy and immunoblot analysis.

Plasmid rescue and sequence analysis of integrants

Human keratinocytes were transfected with pTnori and a helper plasmid expressing either functional (SB) or nonfunctional (mSB) transposase. After 10 days selection, keratinocyte colonies were trypsinized and subcloned at limiting dilution to obtain highly proliferative holoclones.[2,19] Genomic DNA was extracted and digested with *Nhe*I, *Spe*I and *Xba*I. Each of these endonucleases produces compatible cohesive ends and does not cut within the transposon or the plasmid encoded ampicillin gene.[7] Ligations were performed with T4 DNA ligase under dilute conditions, then transformed into DH10B electrocompetent cells followed by replica plating on LB agar containing ampicillin (100 μg/ml) or kanamycin (30 μg/ml). Transposon DNA reisolated from Amp^R/Kan^R bacteria was analyzed by *Hind*III digestion and gel electroporesis. The DNA flanking 5′ and 3′ terminal transposon repeats was sequenced using primers IR-1 (5′-AGATGTCCTAACTGACTTGCC-3′) and IR-2 (5′-GTGGTGATCCTAACTGA CCTT-3′), respectively.

Animal studies

JEB patient and normal control skin was regenerated using primary keratinocytes on CB.17 *scid*/*scid* mice using porcine dermal substrate to avoid immune cross-reactivity of antibodies to laminin 5 as described.[2] The duration of the *in vivo* experiment was 16 weeks; at 4, 8 and 16 weeks postgrafting, human skin tissue was excised and analyzed.

Analysis of protein expression and tissue ultrastructure

Polyclonal antibodies to human laminin 5 and the K140 monoclonal antibody to human laminin β3 (gift of MP Marinkovich)[30] were used to verify expression of both the laminin 5 trimer and full-length laminin β3 via immunoblot analysis. Keratinocyte extracts were prepared and 20 μg of extract protein electrophoresed on a

6% polyacrylamide gel with blots incubated simultaneously with antibodies to BRG1 control[31] for loading, extract quality and transfer efficiency. For immunohistochemistry, 5 μm skin cryosections were fixed and immunostained with laminin 5 antibodies as well as human species-specific antibodies to involucrin (Covance). Sections were also analyzed by immunogold electron microscopy to assess human anchoring filament formation and hemidesomosome ultrastructure.

Acknowledgements

We thank MP Marinkovich for antibody reagents and helpful discussions at all stages of this work. We also thank F Scholl, Z Siprashvili, A Nguyen, N Griffiths, P Bernstein, PB Robbins and other members of the Khavari Laboratory, Mar-kade foundation, Klaus Wolff Klewens Rappersberger.

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