Correction of Laminin-5 Deficiency in Human Epidermal Stem Cells by Transcriptionally Targeted Lentiviral Vectors

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Deficiency of the basement membrane component laminin-5 (LAM5) causes junctional epidermolysis bullosa (JEB), a severe and often fatal skin adhesion defect. Autologous transplantation of epidermal stem cells genetically corrected with a Moloney leukemia virus (MLV)-derived retroviral vector reconstitutes LAM5 synthesis, and corrects the adhesion defect in JEB patients. However, MLV-derived vectors have genotoxic characteristics, and are unable to reproduce the physiological, basal layer–restricted expression of LAM5 chains. We have developed an alternative gene transfer strategy based on self-inactivating (SIN) or long terminal repeat (LTR)-modified lentiviral vectors, in which transgene expression is under the control of different combinations of promoter-enhancer elements derived from the keratin-14 (K14) gene. Analysis in human keratinocyte cultures and in fully differentiated skin regenerated onto immunodeficient mice showed that gene expression directed by K14 enhancers is tissue-specific and restricted to the basal layer of the epidermis. Transcriptionally targeted lentiviral vectors efficiently transduced clonogenic stem/progenitor cells derived from a skin biopsy of a JEB patient, restored normal synthesis of LAM5 in cultured keratinocytes, and reconstituted normal adhesion properties in human skin equivalents transplanted onto immunodeficient mice. These vectors are therefore an effective, and potentially more safe, alternative to MLV-based retroviral vectors in gene therapy of JEB.

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INTRODUCTION

The continuous renewal of the epidermis is sustained by stem cells contained in the epidermal basal layer and in the bulge of hair follicles.[1,2] Upon division, epidermal stem cells produce transit amplifying progenitors that generate all the epithelial components of the skin.[3–5] Basal keratinocytes adhere to the underlying basement membrane through hemidesmosomes. These are multiprotein complexes that include the $a_6\beta_4$ integrin and the basement membrane component laminin-5 (LAM5).[6,7] Mutations in the genes encoding $a_6\beta_4$ integrin or any of the three chains (α_3, β_3, or γ_2) of the LAM5 heterotrimer give rise to a group of autosomal recessive skin adhesion disorders known as junctional epidermolysis bullosa (JEB).[6,8] The severity of JEB depends on the extent of the protein defect, and the consequences may vary from early lethality (the Herlitz forms) to highly disfiguring clinical conditions characterized by blistering, infections, and visual impairment, and an increased risk of skin cancer.[6,8] Current therapeutic approaches to JEB are essentially aimed at controlling infections and maintaining an acceptable quality of life. We have recently shown that gene therapy of LAM5-deficient JEB through transplantation of cultured skin derived from genetically modified epidermal stem cells is feasible, and leads to full functional correction of the disease.[9] However, gene transfer into epidermal stem cells was achieved by transduction with Moloney leukemia virus (MLV)-derived retroviral vectors. This approach has raised serious safety concerns because of the genotoxic risk associated with the insertion of viral long terminal repeats (LTRs) elements into the human genome.[10–12] Development of alternative vector designs, *e.g.*, self-inactivating (SIN) retroviral vectors in which transgene expression is driven by cellular promoters with short-range enhancer activity, is therefore a mandatory prerequisite for future clinical applications, particularly those involving long-lived stem cells.

For gene therapy of LAM5-deficient JEB, restriction of transgene expression to the basal cell layer is an additional required characteristic of the gene transfer vector. In fact, we showed that the MLV LTR directs transcription of the LAM5 β_3 (*LAMB3*) cDNA in all layers of the transduced epidermis, and that, although the synthesis of heterotrimeric LAM5 is restricted to the basal layer because of the absence of the complementary chains, excess intracellular β3 chains were occasionally observed in suprabasal cells also.[9] Accumulation and degradation of unassembled β_3 chains may have unpredictable side effects for the transplanted

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epidermis. Transcriptional targeting of gene expression can be achieved by using a basal cell–restricted regulatory element such as the enhancer/promoter of the keratin-14 (K14) gene. The K14 gene expression is high in the basal layer and is turned off when keratinocytes move to suprabasal compartments.[13] Studies in transgenic mice have shown that a 2.1-kb genomic fragment upstream of the K14 transcription start site is sufficient to reproduce the basal layer–restricted expression of the K14 gene.[14,15] Detailed mapping of this region identified a bipartite enhancer region (−2,000 to −1,300) containing two DNase hypersensitive sites (HS-III and HS-II) that is sufficient to direct transgene expression into the epidermis of transgenic mice when linked to a minimal promoter,[15,16] and a 50-bp region immediately upstream of the TATA box (−97 to −43) containing a putative negative regulatory (NR) element.

In this study, we report the development of transcriptionally targeted, SIN lentiviral vectors in which either the full 2.1-kb K14 enhancer/promoter or a reduced (HS-III+HS-II+NR) enhancer element drives the expression of a reporter [enhanced green fluorescent protein (EGFP)] gene or a *LAMB3* cDNA. The full K14 element was inserted in an internal position into the vector, while the reduced element was introduced into the LTR using an enhancer replacement strategy.[17] Transgene expression was evaluated *in vitro* in both normal and *LAMB3*-deficient human primary keratinocytes, and *in vivo* after xenogeneic transplantation onto immunodeficient mice. All vectors were able to transduce long-term repopulating epidermal stem cells, to restrict transgene expression to the basal layer of the epidermis *in vivo*, and to restore LAM5 synthesis and adhesion properties in *LAMB3*-deficient keratinocytes both *in vitro* and *in vivo*. These results indicate that transcriptionally targeted lentiviral vectors are an effective alternative to conventional retroviral vectors for use in gene therapy of JEB.

RESULTS

Construction and testing of SIN lentiviral vectors containing K14 regulatory elements

In order to restrict transgene expression to the basal layer of the epidermis, we developed transcriptionally targeted lentiviral vectors in which gene expression is under the control of different regulatory elements of the human K14 gene. In the first design, expression of the EGFP gene was driven by a 2.1-kb region upstream of K14 containing the promoter, and a regulatory region known to restrict transgene expression to the epidermal basal layer in transgenic mice.[14] The expression cassette was inserted in a U3-deleted (−418 to −18) SIN lentiviral vector containing the human immunodeficiency virus (HIV) central polypurine tract and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE)[18] (K.G vector in **Figure 1**). In a control vector (P.G in **Figure 1**), EGFP expression was under the control of a constitutive phosphoglycerokinase promoter. In a second class of vectors, the EGFP gene was placed under the control of a modified viral LTR in which the U3 enhancer (−418 to −40) was replaced with a 500-bp fragment containing the K14 HS-III and HS-II enhancers (HS.G in **Figure 1**), or with a 600-bp fragment containing HS-III, HS-II, the entire spacer between them, and the NR upstream promoter region (HS.NR.G in **Figure 1**). All four vectors were pseudotyped with vesicular stomatitis virus G by a second-generation lentiviral packaging system[19] at titers ranging from 10^6 to 10^8 transducing units/ml, as determined by infection of HaCaT (a human keratinocyte cell line) or HeLa cells with serial dilutions of viral supernatants, and by fluorescence-activated cell sorting analysis of EGFP expression. Titers measured using real-time PCR gave estimates consistent with those obtained in HaCaT cells. Titers calculated in HeLa cells were consistently lower, as expected when using vectors based on tissue-specific regulatory elements.

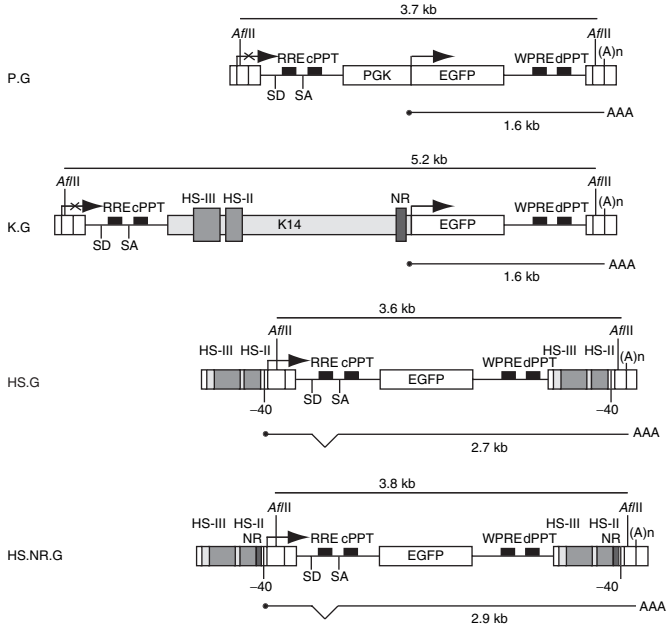

Figure 1 Schematic map of the enhanced green fluorescent protein (EGFP)-expressing, human immunodeficiency virus 1 (HIV1)-derived lentiviral vectors in their proviral forms. In the first two vectors, the transgene is under the control of either the constitutive phosphoglycerokinase (PGK) promoter (P.G) or the 2.1-kb K14 enhancer/promoter element (K.G). The position of the HS-III and HS-II hypersensitive sites and of the upstream negative regulatory (NR) region in the K14 element are indicated by dark gray boxes. Both vectors carry a deletion of the long terminal repeat (LTR) U3 enhancer/promoter (−418 to −18). In the last two vectors, the U3 enhancer (−418 to −40) was replaced by either a 500-bp fragment of the K14 element containing the HS-III and HS-II sites (HS.G) or by a 600-bp fragment containing the HS-III and HS-II sites with their natural spacer plus the NR region (HS.NR.G). Both vectors maintain the HIV TATA box promoter. Arrows indicate active transcription start sites. Crossed arrows indicate the disabled LTR promoters in the P.G and K.G vectors. Restriction sites used for Southern blot analysis (*Afl*II), splice donor (SD) and acceptor (SA) sites, Rev-responsive element (RRE), central and distal polypurine tract (cPPT and dPPT), polyadenylation site [(A)n], and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) are indicated. For each vector, the sizes of the genomic provirus (upper bar) and of the spliced proviral transcript (lower bar) are shown.

Transduction of human keratinocytes by transcriptionally targeted lentiviral vectors

Fluorescence-activated cell sorting analysis of EGFP expression in HaCaT cells transduced at different multiplicities of infection (MOIs) (0.1, 1, 10, 25, and 50) showed a linear increase of the mean fluorescence intensity for all vectors (**Figure 2a**). At all MOIs, the K.G vector showed the highest GFP expression levels (~50% of those of the control P.G vector), while the

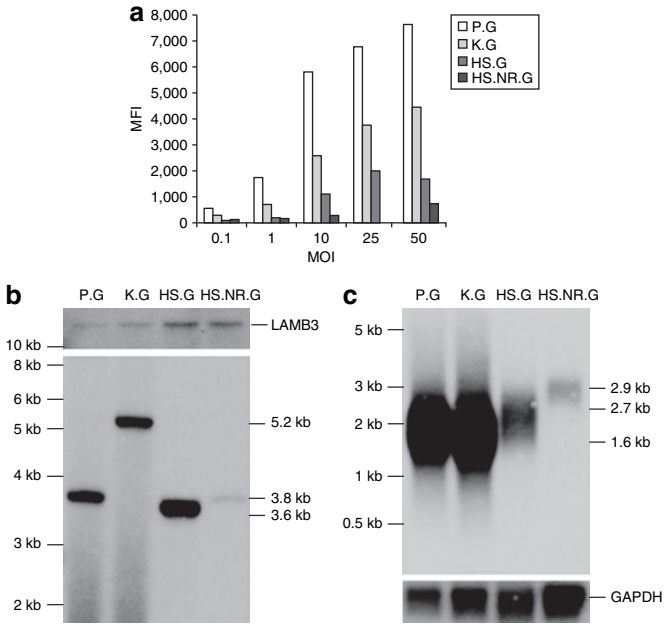

Figure 2 Transduction of HaCaT epithelial cells by the lentiviral vectors shown in Figure 1. (**a**) Mean fluorescence intensity (MFI) of cells transduced at different multiplicities of infection (MOIs) evaluated by fluorescence-activated cell sorting analysis 6 days after transduction. (**b**) Southern blot analysis of genomic DNA extracted from HaCaT cells transduced with the four vectors at an MOI of 10, digested with *Afl*II, and hybridized to a green fluorescent protein (GFP) probe (lower panel) or to a laminin-B3 (LAMB3) probe (upper panel) to normalize for loaded DNA. Molecular weight markers are indicated on the left, and the size of each provirus (in kb) is indicated on the right. (**c**) Northern blot analysis of poly(A)$^+$ RNA extracted from HaCaT cells transduced at an MOI of 10, and hybridized to a GFP probe (upper panel) or to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (lower panel) to normalize for mRNA content. Molecular markers are indicated on the left, and the size of each spliced proviral transcript (in kb) is indicated on the right.

LTR-modified vectors showed consistently lower levels of expression (**Figure 2a**). Southern blot analysis of genomic DNA from cells transduced at an MOI of 10 (62–88% transduction efficiency) and restricted with *Afl*II showed that transduced cells harbored stably integrated vectors of the expected size with an estimated average vector copy number per transduced cell of 11.5–17.7 for P.G, K.G, and HS.G, and 4.7 for HS.NR.G, after normalization with a probe for the endogenous LAMB3 gene (**Figure 2b**). Real-time quantitative PCR gave comparable vector copy number estimates, ranging from 15.6 to 21.7 for P.G, K.G, and HS.G, and 2.2 for HS.NR.G. Northern blot analysis of RNA from the same cells showed accumulation of a single vector–derived mRNA of the expected size for all vectors (a 1.6-kb transcript for the internal expression cassettes, and fully spliced, 2.7- and 2.9-kb transcripts for the LTR-modified vectors, **Figure 2c**). Transcripts from P.G and K.G vectors accumulated at much higher levels than those from the HS.G and HS.NR.G vectors respectively, as estimated by phosphorimaging after normalization for glyceraldehyde-3-phosphate dehydrogenase mRNA content (**Figure 2c**). These data show that P.G and K.G vectors, featuring internal expression cassettes, accumulate more transcripts per integrated provirus than the LTR-modified HS.G and HS.NR.G. Integration of HS.NR.G was much less efficient when compared with all the other vectors, possibly because of the incorporation of a larger DNA fragment into the LTR.

The four vectors were used at different MOIs to transduce subconfluent human primary foreskin–derived keratinocytes cultured onto a feeder layer of lethally irradiated 3T3-J2 cells. Fluorescence-activated cell sorting analysis of EGFP expression showed that transduction efficiency reached 60–80% at an MOI of 10, with the exception of the HS.NR.G vector which transduced only 15% of the cells, and showed a trend similar to that observed in HaCaT cells in terms of correlation between mean fluorescence intensity and MOI (**Figure 3**). At MOI 10, the average vector copy number per transduced cell was lower than that observed in HaCaT cells, ranging from 0.5 for HS.NR.G to 6.6 for K.G, as analyzed using real-time PCR (see legend to **Figure 3**). Keratinocytes transduced at MOIs of 10 and 25 were also analyzed for their clonogenic capacity, using a colony-forming efficiency assay. Clonogenic keratinocytes were present in normal numbers, and were transduced at an efficiency comparable to that of bulk cultures (data not shown), thereby indicating that transduction with lentiviral vectors does not alter the clonogenic capacity of primary human keratinocytes.

Keratinocytes transduced with lentiviral vectors generate normal human skin *in vivo*

In order to analyze the transcriptional activity of the targeted vectors in stratified human skin, we used an *in vivo* model that allows long-term regeneration of genetically modified skin after xenotransplantation onto immunodeficient (*nu/nu*) mice. Using a previously described procedure[20,21] (**Figure 4**), human skin implants were constructed *ex vivo* by seeding keratinocytes transduced with all lentiviral vectors on a dermal equivalent composed of a fibroblast-containing fibrin matrix, and grafted onto immunodeficient mice. Overall, four mice received transplants of untransduced skin, and four groups of six mice received transplants of skin obtained from keratinocytes transduced with the P.G, K.G, HS.G, and HS.NR.G vectors, respectively. Samples of the transplanted epidermis were examined 8–22 weeks after grafting. Differentiation was assessed morphologically and by the expression of endogenous K14 protein, as evaluated by immunofluorescence on untransduced and transduced skin sections. At the latest time points (14–22 weeks), the regenerated skin appeared fully differentiated, with a well-developed *stratum granulosum* and a typical basket-weave *stratum corneum* (**Figure 5**). The expression of K14 was restricted to the basal layer and occasionally to the first suprabasal layer in untransduced regenerated human skin (data not shown) as well as in transduced regenerated human skin (**Figure 5a–d**, right panels). In all the groups of transplanted mice, EGFP$^+$ human skin persisted at least 22 weeks after transplantation, thereby demonstrating that lentiviral vectors transduce repopulating epidermal stem cells and do not affect their *in vivo* regeneration and differentiation capacity.

Transcriptionally targeted lentiviral vectors restrict transgene expression to the basal layer of human epidermis *in vivo*

Epifluorescent illumination of the transplanted zone allowed *in vivo* monitoring of the genetically modified graft at every

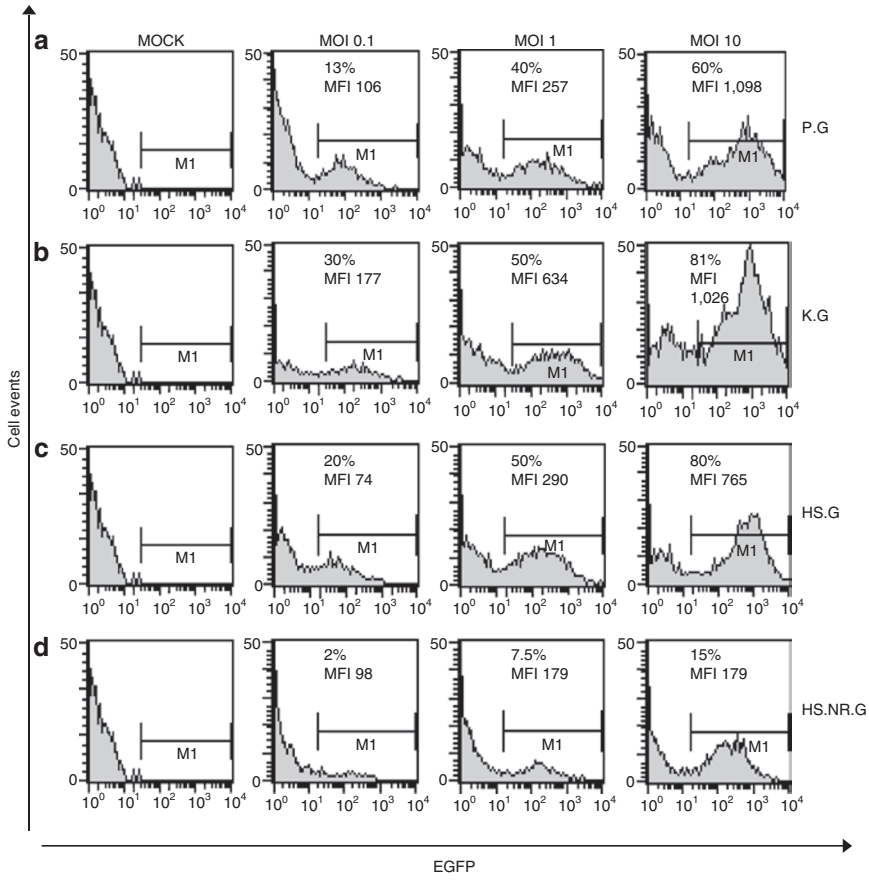

Figure 3 Analysis of enhanced green fluorescent protein (EGFP) expression in human primary keratinocytes transduced with the lentiviral vectors shown in Figure 1. Subconfluent human primary keratinocytes were transduced at multiplicities of infection (MOIs) of 0.1, 1.0, and 10, and EGFP expression was evaluated by fluorescence-activated cell sorting analysis 2 weeks after transduction. In all the graphs, transduction efficiency (% of GFP^+ cells) and mean fluorescence intensity (MFI) of GFP^+ cells are indicated. The average vector copy numbers per transduced cell were 0.8, 2.0, and 5.8 for the P.G vector, 1.0, 2.4, and 6.6 for the K.G vector, 0.7, 2.5, and 6.7 for the HS.G vector, and 0.3, 0.3, and 1.1 for the HS.NR.G vector.

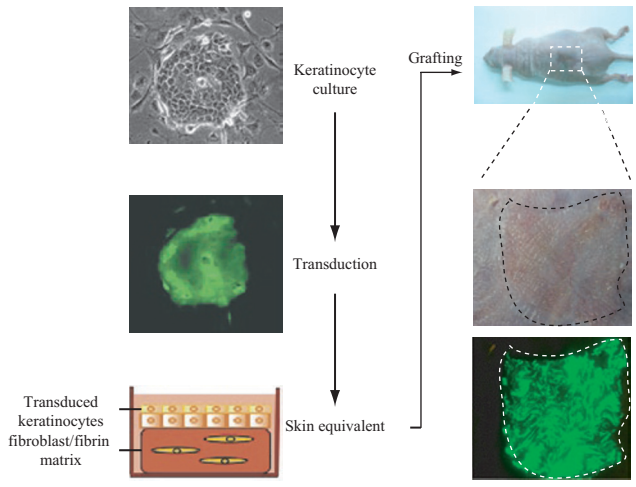

Figure 4 Transplantation of human transduced epidermal keratinocyte-derived skin equivalents onto immunodeficient mice. Skin equivalents were derived by seeding keratinocytes transduced by the lentiviral vectors shown in **Figure 1** on a fibroblast-containing fibrin matrix. These were allowed to form a stratified epithelium in culture, and grafted onto the backs of immunodeficient (*nu*/*nu*) mice. Expression of enhanced green fluorescent protein in the transduced epidermis was monitored *in vivo* under a UV lamp. The skin represented in the picture was derived from keratinocytes transduced with the HS.NR.G lentiviral vector.

time point examined. Grafts of transduced keratinocytes were harvested at the different time points, sectioned, and analyzed for expression of EGFP protein using fluorescence microscopy and EGFP mRNA by *in situ* hybridization. The P.G vector, for which EGFP is under the control of the constitutive phosphoglycerokinase promoter, led to accumulation of EGFP transcripts and protein in all layers of the regenerated/engrafted epidermis (**Figure 5a**). In comparison, the K.G vector, for which EGFP is under the control of the full K14 enhancer/promoter, drove EGFP synthesis only in the basal layer of the epidermis in all analyzed mice, as indicated by colocalization of EGFP protein (**Figure 5b**, left panel) and transcripts (**Figure 5b**, center panel) in the same layers that express the endogenous K14 (**Figure 5b**, right panel). The HS.G vector, driving EGFP expression from the viral LTR promoter regulated by the K14 HS-III+II elements, showed a similar restriction to the $K14^+$ basal keratinocytes in all grafts (**Figure 5c**). However, EGFP transcripts were occasionally detected also in $K14^-$ cells of the suprabasal layers, with some intragraft variability (**Figure 5c**). Finally, EGFP expression from the HS.NR.G vector, containing the K14 NR element upstream of the HIV promoter, was always fully restricted to the basal keratinocytes, with a pattern indistinguishable from that observed for the K.G vector (**Figure 5d**).

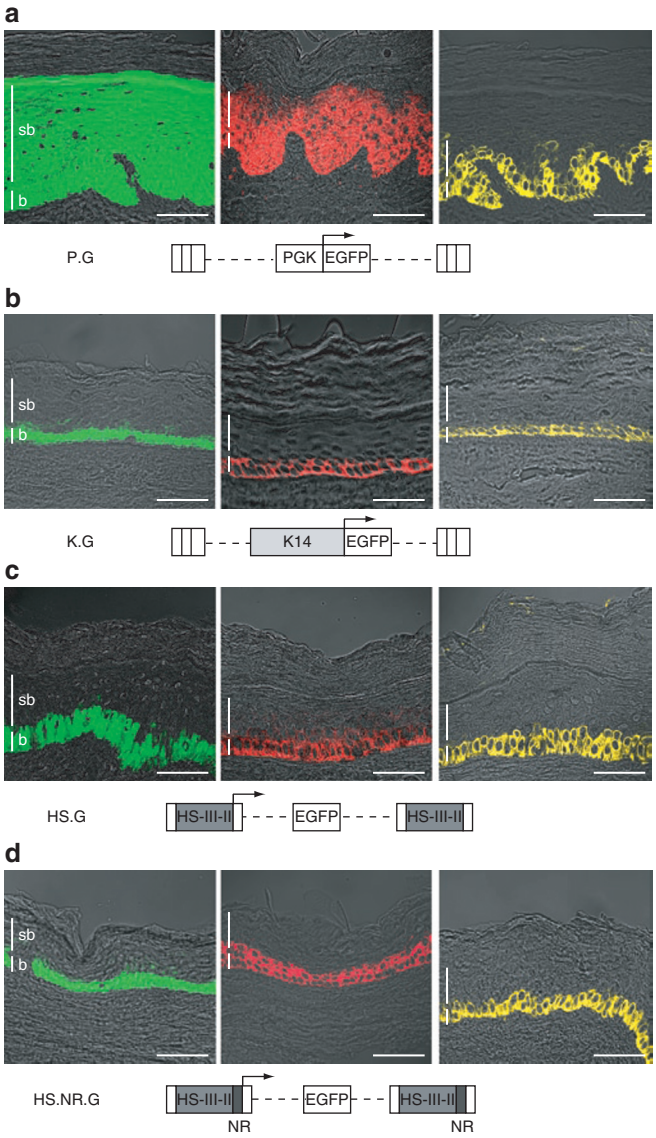

Figure 5 Restriction of transgene expression in xenotransplants of transduced human epidermis. (**a–d**) Immunofluorescence analysis of enhanced green fluorescent protein (EGFP) (green, left panels) and endogenous human K14 protein (yellow, right panels), and *in situ* hybridization analysis of EGFP RNA (red, center panels) in histological cryosections of regenerated epidermis transduced with the lentiviral vectors shown in **Figure 1**, and analyzed at 12 (HS.G), 20 (K.G and HS.NR.G), and 22 (P.G) weeks after transplantation onto immunodeficient mice. A schematic map of each vector is shown under each group of panels. In all the cases, regenerated human skin appeared fully differentiated, with a well-developed *stratum granulosum* and a typical basket-weave *stratum corneum*. (**a**) In skin transduced with the P.G vector, EGFP protein and RNA are expressed in all the layers. In skin transduced with (**b**) the K.G vector, (**c**) the HS.G vector, and (**d**) the HS.NR.G vector, expression of EGFP was restricted to keratinocytes of the basal (b, white bar) layer, and occasionally to scattered cells in the first suprabasal (sb, white bar) layer, with a pattern indistinguishable from that observed for the endogenous K14 protein. Horizontal scale bar = 100 µm. PGK, phosphoglycerokinase.

Lentiviral vector transduction reconstitutes normal levels of LAMB3 in LAMB3-deficient keratinocytes

In order to examine whether transgene expression levels from transcriptionally targeted lentiviral vectors are sufficient to restore

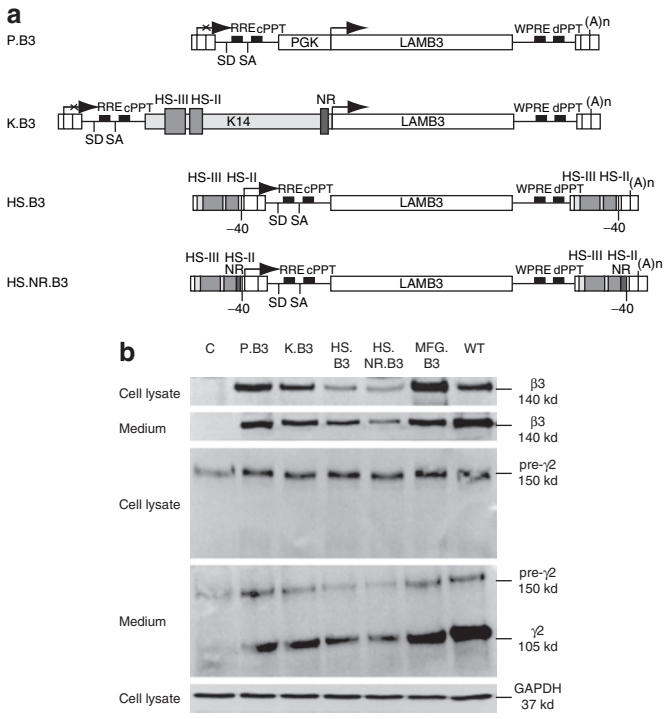

Figure 6 Correction of laminin-B3 (LAMB3) deficiency by lentiviral vector–mediated gene transfer. (**a**) In the first two vectors, the *LAMB3* cDNA is under the control of either the phosphoglycerokinase (PGK) promoter (P.B3) or the 2.1-kb K14 enhancer/promoter element (K.B3). The positions of the HS-III, HS-II, and negative regulatory (NR) regions in the K14 element are indicated by dark gray boxes. Both vectors carry a deletion of the long terminal repeat (LTR) U3 enhancer/promoter (–418 to –18). In the last two vectors, the U3 enhancer (–418 to –40) was replaced with either a 500-bp fragment of the K14 element containing the HS-III and HS-II sites (HS.B3) or with a 600-bp fragment containing the HS-III and HS-II sites with their natural spacer plus the NR region (HS.NR.G). (**b**) Western blot analysis of the expression of the β_3- and γ_2-chain of LAM5 in whole-cell lysates and culture media from normal keratinocytes [wild type (WT)] and *LAMB3*-deficient cells from a junctional epidermolysis bullosa patient, before C and after transduction with the four lentiviral vectors and the MFG-*LAMB3* γ-retroviral vector (MFG.B3) used in the previously described clinical trial.[9] Blots were immunostained with LAM5 β_3- and γ_2-chain specific antibodies. Cell lysates were immunostained with an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to normalize for protein loading. All vectors restore full expression of β3 chains and secretion of mature LAM5, as indicated by the presence of the processed, 105-kd form of the γ_2-chain in the culture medium. The average vector copy number was 8.4 for P.B3, 5.0 for K.B3, 4.2 for HS.B3, 1.0 for HS.NR.B3, and 3.1 for MFG. cPPT, central polypurine tract; dPPT, distal polypurine tract; RRE, Rev-responsive element; SA, splice acceptor; SD, splice donor; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

LAM5 synthesis in keratinocytes from JEB patients, we replaced the EGFP gene with a *LAMB3* cDNA in all vector designs. The resulting vectors (P.B3, K.B3, HS.B3, and HS.NR.B3, **Figure 6a**) were used in transducing keratinocytes derived from a patient affected by a nonlethal form of LAMB3-deficient JEB. These keratinocytes are unable to synthesize heterotrimeric LAM5, have a decreased colony-forming capacity, and give rise to skin cultures with impaired adhesion properties.[9] Subconfluent keratinocytes were cultured onto a feeder layer of lethally irradiated 3T3-J2 cells, and transduced with all lentiviral vectors at an MOI of 25, and with the MFG-*LAMB3* γ-retroviral vector used in the

pilot clinical trial of gene therapy of JEB.[9] Transduction efficiency exceeded 80% for all vectors except HS.NR.B3 (efficiency 43%), as indicated by immunofluorescence analysis of cytoplasmic LAMB3 (data not shown). Whole-cell lysates and culture media were analyzed for the presence of intracellular and secreted LAM5, using western blotting with antibodies specific for the LAM5 β_3 and γ_2 chains. All vectors restored expression of β_3 chains in cell lysates and LAM5 secretion, as indicated by the presence of β_3 chains and of the processed, 105-kd form of the γ_2-chain in the culture medium. In particular, the P.B3 and K.B3 vectors secreted β_3 chains at levels comparable to those of JEB keratinocytes transduced with the MFG-*LAMB3* vector and control keratinocytes from a healthy donor (**Figure 6b**).

Transcriptionally targeted LAMB3 expression restores the adhesion properties of epidermal stem cell–derived skin *in vivo*

In order to test the therapeutic potential of transcriptionally targeted *LAMB3* gene expression, we transduced patient-derived, LAMB3-deficient keratinocytes with the K.B3 vector at an MOI of 25, and generated transplantable skin equivalents, as described earlier. Transduction efficiency reached 55% as assessed by immunofluorescence with an anti-β_3 antibody (data not shown). Two groups of four immunodeficient mice each were transplanted with genetically corrected skin and with untransduced, control skin, respectively. Untransduced skin grafts were lost on all the mice within 4 weeks, while transduced skin equivalents grafted successfully in three of the four animals. The first mouse was killed 9.5 weeks after transplantation, and the clearly recognizable graft (**Figure 7a**) was analyzed for *LAMB3* transcript and protein expression by *in situ* hybridization and immunohistochemistry. At low magnification, vector RNA expression clearly marked the edges between human and murine skin (**Figure 7b**). At higher magnification, human skin appeared firmly adherent on top of a basal lamina staining positive for human LAM5 β_3 chains (**Figure 7c**). All basal layer keratinocytes stained positive for human K14 and *LAMB3* RNA expression (**Figure 7d** and **e**). These data show that a restricted *LAMB3* transgene is capable of restoring the adhesion properties of LAMB3-deficient skin derived from transduced epidermal stem/progenitor cells.

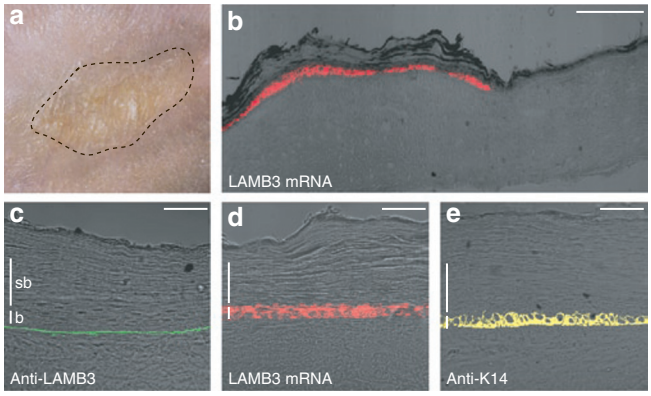

Figure 7 Corrrection of laminin-B3 (LAMB3) deficiency in vivo by lentiviral vector–mediated gene transfer. (**a**) Skin equivalents were derived by seeding keratinocytes from a LAMB3-deficient junctional epidermolysis bullosa patient after transduction with the K.B3 lentiviral vector, and grafted onto immunodeficient (*nu*/*nu*) mice. The picture shows one of the grafts 9 weeks after transplantation. (**b**) *In situ* hybridization analysis of LAMB3 mRNA (red) in histological cryosections of K.B3-transduced regenerated epidermis at the junction between human and murine skin 9.5 weeks after transplantation. Bar = 500 µm. (**c–e**) Immunofluorescence and *in situ* hybridization analysis of the LAM5 β3 chains (green), LAMB3 mRNA (red), and endogenous human K14 (yellow) in histological cryosections of K.B3-transduced regenerated epidermis 9 weeks after transplantation. Basal (b) and suprabasal (sb) skin layers are indicated by vertical white bars. Horizontal scale bar = 100 µm.

DISCUSSION

Transplantation of autologous, genetically corrected epidermal stem cells may provide a cure for many forms of skin adhesion disorders. Recently, in a pilot clinical trial, we provided proof of principle for the efficacy of this technology in reversing the phenotypic consequences of LAM5-deficient JEB.[9] The study was based on the use of a γ-retroviral vector driving *LAMB3* gene expression under the control of a wild-type MLV LTR. This type of vector has been associated with the occurrence of malignancies in clinical trials of gene therapy for severe combined immunodeficiency,[22] and of potentially dangerous clonal imbalance in patients treated for chronic granulomatous disease.[23] Recent studies have indeed shown that MLV-based retroviral vectors have a high tendency to target growth-controlling genes and proto-oncogenes, at least in hematopoietic cells,[24] and that insertion of the MLV LTR into the genome has a very high chance of deregulating neighboring genes, independent of their function.[25] Although there is no evidence that genetic modification of nonhematopoietic stem cells may be associated with similar risks, development of alternative gene transfer vectors is strongly recommended, or required, by regulatory authorities in the Europe and United States. In this report, we show that SIN lentiviral vectors carrying tissue-specific promoter/enhancer elements are able to transduce repopulating epidermal stem cells, drive basal layer–restricted gene expression, and correct the adhesion properties of LAMB3-deficient skin grafts *in vivo* in a xenogeneic transplantation model, thereby providing an effective alternative to MLV-based vectors in gene therapy for skin adhesion disorders. A SIN design and the use of cellular enhancer elements instead of viral LTRs to drive gene expression are expected to decrease the chances of insertional gene activation and oncogenesis in repopulating cells. Recent studies in a murine model *in vivo* have shown that this is indeed the case at least for the hematopoietic system.[26] Further studies are necessary for defining the safety profile of SIN lentiviral vectors in epidermal keratinocytes.

In order to restrict gene expression to the basal layer of the transplanted skin, we chose a previously described 2.1-kb genomic fragment that includes the K14 promoter and its upstream regulatory elements. Studies in transgenic mice had shown that this element is sufficient to reproduce the basal layer–restricted expression of the K14 gene.[14,15] Detailed mapping of this region identified a bipartite enhancer that contains two DNase hypersensitive sites (HS-III and HS-II) and is sufficient to direct transgene expression into the epidermis of transgenic mice when linked to a minimal promoter.[15,16] This enhancer contains binding sites for tissue-specific as well as nonspecific transcription factors such as Ets, Ap-1, Ap-2, Sp1/Sp3, Skn-1, ΔNp63α, and GATA, which

cooperate to achieve basal keratinocyte–specific transcriptional activity.[15,16,27] In addition, a 50-bp region immediately upstream of the TATA box (−97 to −43) contains a putative NR element with ill-defined function. The NR element contains sequences necessary for POU domain factor–mediated repression of transcription *in vitro* (most probably through interference with the function of coactivators like CBP/p300),[28] and putative responsive elements for glucocorticoid, retinoic acid, and thyroid hormone receptors.[29,30] In our somatic transgenesis model, the full 2.1-kb enhancer/promoter is able to reproduce the entire K14-specific restriction to basal layer cells. A synthetic enhancer composed of the HS-III and HS-II regions is still capable of restricting gene expression, although with occasional leaking into the first or second layer of suprabasal cells. Interestingly, addition of the NR region abolishes any leakiness in gene expression, suggesting that this region might mediate transcriptional repression in suprabasal cells.

The synthetic enhancers were used in a heterologous context, to drive expression of the HIV promoter within the lentiviral LTR. This vector design (U3 enhancer-replaced) has been previously shown to increase efficiency and stability of protein expression, because of the use of the spliced major retroviral genomic transcript rather than of an unspliced internal transcript to drive expression of the transgene.[17] Indeed, the HS.G and HS.NR.G vectors showed a good performance in terms of number of transcripts per integrated copy of the vector, although their transduction capacities in terms of average vector copy number per transduced cell were consistently lower than those of vectors based on an internal cassette (K.G). Nevertheless, both LTR-modified vectors produced enough LAMB3 protein to correct the LAM5 defect in patient-derived primary keratinocytes, although only incompletely as compared to the internal cassette-based K.G vector and to the LTR-driven γ-retroviral vector that were used in the pilot clinical trial. A potential safety concern of this design derives from the presence of two copies of the enhancer elements, which could result in a higher *trans*-activating activity once integrated into the target cell genome. In addition, superinfection by a HIV could theoretically result in the mobilization and spreading of the integrated vector. However, this would hardly be a concern in epidermal stem cell–derived tissues, because they cannot be infected by HIV *in vivo*.

Transduction of patient-derived LAMB3-deficient keratinocytes and analysis of LAM5 synthesis and secretion *in vitro* showed that all vectors direct enough β_3-chain synthesis to correct the genetic defect, to an extent similar to the one shown by the MFG-LAMB3 vector used in the pilot clinical trial. Transplantation of skin equivalents derived from epidermal stem cells transduced with one of the targeted vectors (K.B3) showed full functional recovery of the graft adhesion properties. Interestingly, although the keratinocyte culture used for building the epidermal grafts was transduced at ~50% efficiency, all the cells in the graft basal layer stained positive for vector-derived transcripts, thereby indicating an *in vivo* selection of genetically corrected cells. This phenomenon, which could not be observed in the clinical trial (the transduction efficiency of the MFG-LAMB3 vector was >90%), suggests that uniform transduction of keratinocyte culture may not be a mandatory requirement for clinical application. Transduction at lower MOIs would result in less toxicity and a lower number of integrated proviruses, thereby improving the overall safety profile of the genetic modification.

In conclusion, transcriptionally targeted lentiviral vectors based on K14 enhancer/promoter elements are capable of infecting epidermal stem cells, restricting their expression to the basal layer of transplanted epidermis, and producing enough LAMB3 protein to correct the genetic defect in patient-derived cells *in vitro* and *in vivo*. These vectors may soon replace MLV-derived retroviral vectors in clinical studies aimed at the correction of inherited skin adhesion disorders.

MATERIALS AND METHODS

Lentiviral vectors and vector production. All lentiviral vectors were built in the framework of the SIN pRRLsin-18.pptCMV-GFPwpre vector.[31] In the P.G vector, the EGFP gene was placed under the control of the human phosphoglycerokinase promoter. In the K.G vector, the phosphoglycerokinase promoter was replaced by an *Ava*I fragment containing the 2.1-kb promoter/enhancer of the human K14 gene.[14] The HS.G and HS.NR.G vectors both carried a deletion in the HIV-1 LTR (−418 to −40) that removes the U3 enhancer but not the minimal promoter, generated by *Eco*RV and *Ban*II digestion and religation. In the HS.G vector, the U3 enhancer was replaced with a 500-bp fragment containing the HS-III (*Bst*XI/*Nsp*I, −1,760 to −1,550) and HS-II (*Ava*II/*Sap*I, −1,450 to −1,325) K14 enhancer elements.[15,16] In the HS.NR.G vector, an additional PCR-amplified 50-bp region (−97 to −43) immediately upstream of the TATA box of the K14 promoter was added between the HS-III+HS-II region and the LTR promoter. A second set of vectors was built by replacing the EGFP cDNA with the 3.8-kb *Xba*I/*Xmn*I fragment encoding the full-length LAMB3 cDNA in all the vectors described above, to obtain the P.B3, K.B3, HS.B3, and HS.NR.B3 vectors.

Viral stocks pseudotyped with the vesicular stomatitis virus G protein were prepared by transient cotransfection of 293T cells using a three-plasmid system (the transfer vector, the pCMVΔR8.74 encoding Gag, Pol, Tat, and Rev, and the pMD.G plasmid encoding vesicular stomatitis virus G), as previously described.[19] Viral preparations were concentrated by ultracentrifugation to increase titer. Viral titers were determined by transduction of HaCaT cells with serial dilution of the vector stocks in the presence of 8 μg/ml polybrene, and found to range between 10^7 and 10^8 transducing units/ml. Transduction efficiency was determined by flow cytometry for EGFP vectors, and by real-time quantitative PCR for LAMB3 vectors. Briefly, genomic DNA from ~10^6 transduced cells was isolated 21 days after infection using the QIAmp DNA Mini Kit (Qiagen, Valencia, CA), serially diluted, amplified with proviral-specific primers (forward primer 5′-TGAAAGCGAAAGGGAAACCA-3′; reverse primer 5′-CCGTGCGCGCTTCAG-3′) and detected using a specific fluorescent probe (5′-FAM-AGCTCTCTCGACGCAGGACTCGGC-MGB-3′) on an ABI Prism 7900 machine (Applied Biosystems, Foster City, CA). Primers and probes were synthesized by Applied Biosystems. The TaqMan Ribosomal RNA Control Reagents kit was used for normalization. A standard curve was designed through coamplification of serial dilutions of genomic DNA from ACH2 cells containing one HIV-1 copy per genome (AIDS National Reagent Program, National Institutes of Health, Bethesda, MD).

Cell culture. Human HaCaT cells were maintained in Dulbecco's modified Eagle's medium (EuroClone, Pavia, Italy) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Swiss mouse 3T3-J2 cells (a kind gift from Howard Green, Harvard Medical School, Boston, MA) were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 50 IU/ml penicillin–streptomycin, and 4 mmol/l glutamine. JEB skin keratinocytes were obtained from palm biopsies taken from a 38-year-old male patient affected by nonlethal, LAMB3-deficient JEB, as previously described.[9] Primary normal or JEB keratinocytes were plated

onto lethally irradiated 3T3-J2 cells and cultured in keratinocyte growth Kno medium, a Dulbecco's modified Eagle's medium and Ham's F12 media mixture (2:1) containing fetal calf serum (10%), penicillin–streptomycin (1%), glutamine (2%), insulin (5 μg/ml), adenine (0.18 mmol/l), hydrocortisone (0.4 μg/ml), cholera toxin (0.1 nmol/l), and triiodothyronine (2 nmol/l). After 3 days, Kno was replaced and Kc medium (Kno medium containing 10 ng/ml EGF) was added to the culture. Keratinocytes were trypsinized at subconfluency and replated onto a new feeder layer.

Transduction of keratinocytes. Lentiviral transduction of HaCaT cells was performed at different MOIs by spinoculation (1 round at 1,800 rpm for 45 minutes) in the presence of 8 μg/ml polybrene. On day 6 after transduction, the cells were harvested and analyzed by flow cytometry for GFP expression. Subconfluent normal or JEB primary skin keratinocytes were trypsinized and 10^5 cells were resuspended into 2 ml of Kno medium containing the different lentiviral vectors at MOIs ranging from 1 to 50, in the presence of 8 μg/ml polybrene. The transduction mixture containing keratinocytes, vector, and polybrene was then plated onto lethally irradiated 3T3-J2 cells. The medium was replaced after 6–7 hours. Transduced keratinocytes were grown to confluence, trypsinized, and replated onto new feeder layers for further analysis. JEB keratinocytes were also transduced with the MLV-derived MFG-LAMB3 retroviral vector, as previously described.[9]

Southern and northern blot analysis. Genomic DNA was extracted from 1×10^6 to 5×10^6 cells using a QIAmp DNA Mini Kit (Qiagen, Valencia, CA), digested overnight with *Afl*II, run in 10 μg aliquots on a 0.8% agarose gel, transferred to a nylon membrane (Duralon, Stratagene, La Jolla, CA) by Southern capillary transfer, and probed with 2×10^7 cpm ^{32}P-labeled GFP or LAMB3 probe. Total cellular RNA was extracted by guanidine-isothiocyanate, poly(A)-selected by oligo(dT)-cellulose chromatography, size fractionated on 1% agarose-formaldehyde gel, blotted onto nylon membranes, and hybridized to 10^7 cpm of ^{32}P-labeled GFP and glyceraldehyde-3-phosphate dehydrogenase probes, in accordance with standard techniques.[32]

Western blot analysis. Subconfluent keratinocytes were incubated in serum-free medium for 24–48 hours at 37 °C. The medium was collected, concentrated by membrane filtration (Amicon Ultra-4, "50,000 molecular weight cutoff"; Millipore, Billerica, MA), and frozen. The cells were lysed in RIPA buffer (50 mmol/l Tris–HCl, 150 mmol/l NaCl, 1% deoxycolate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.2% sodium azide, pH 7.5 containing protease inhibitors) for 30 minutes on ice, and the amount of protein was determined using Bradford assay (Bio-Rad, Hercules, CA). Medium and cell lysates (40–50 μg) were run on a 6% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (80 V, 2–3 hours) gel, transferred onto polyvinylidene fluoride filters (Immobilon-P; Millipore, Billerica, MA), and immunoblotted (100 mA at 4 °C, overnight) using either a polyclonal antibody against the human LAM5 β3-chain (sc-7651, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) or a mouse monoclonal antibody against the LAM5 γ_2-chain (clone D4B5, 1:100; Chemicon, Temecula, CA). The same lysates (50 μg) were also run on a 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and immunoblotted using a rabbit polyclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (1:500, sc-25778; Santa Cruz Biotechnology, Santa Cruz, CA) for protein loading normalization. Protein detection was carried out using a chemiluminescent labeling detection reagent (ECL; GE Healthcare, Milan, Italy).

***Regeneration of human epidermis* in vivo.** A fibrin–fibroblast matrix was prepared as previously described,[20] and used as the dermal component of the artificial skin. The fibrinogen–fibroblasts mixture was placed on 35-mm wells in a 6-well culture plate (Transwell; Costar, Cambridge, MA) and allowed to clot at 37 °C for 2 hours. Untransduced or EGFP$^+$ keratinocytes were then seeded on the fibrin matrix to form the epidermal layer of the artificial skin. Cultures were manually detached from the wells and placed orthotopically on the backs of immunodeficient mice, as previously described.[21] Briefly, the mice were aseptically cleansed, and full-thickness 35-mm circular wounds were created on the dorsum of each mouse to match skin equivalents. Devitalized mouse skin was used as a biological bandage. Grafting was performed under sterile conditions on 6–7-week-old female *nu/nu* mice (Janvier) housed under pathogen-free conditions for the duration of the experiment. Successfully engrafted mice were killed by CO_2 asphyxiation at 8–22 weeks after the grafting. Skin grafts were excised along with ~2 mm of surrounding mouse skin, and either processed for routine histology or fixed in 4% buffered paraformaldehyde (pH 7.4) for 40 minutes at 4 °C, and then embedded in optimal cutting temperature. All experimental procedures were performed in accordance with European and Spanish laws and regulations (European Convention 123, Use and Protection of Vertebrate Mammals in Experimentation and Other Scientific Purposes, Spanish RD 223/88; and OM 13-10-89 of the Ministry of Agriculture, Food, and Fisheries, Protection and Use of Animals in Scientific Research and Internal Biosafety and Bioethics Guidelines).

Immunofluorescence and immunohistochemistry. The expression levels of GFP, K14, and LAM5 β_3 chains were analyzed in paraformaldehyde-fixed samples embedded in optimal cutting temperature, frozen, and sectioned. Five- to seven-micrometer sections were analyzed for GFP expression by indirect immunofluorescence, or were permeabilized (0.5% Triton X-100 in phosphate-buffered saline), coated with 0.5% bovine serum albumin/phosphate-buffered saline for 1 hour at room temperature, and incubated with a 1:100 dilution of either a rabbit monoclonal antibody against human cytokeratin-14 (clone ID: EP1612Y; Epitomics, Burlingame, CA), or the K140 monoclonal antibody against LAM5 β_3 for 1 hour at 37 °C, followed by a 60-minute incubation at room temperature with a 1:100 dilution of a rhodamine-conjugated secondary antibody.

In situ *RNA hybridization.* Digoxigenin-labeled cRNAs were synthesized in accordance with the manufacturer's instructions (DIG RNA Labeling kit; Roche, Monza, Italy). Primers carrying the Sp6/T7 promoter sequences (MWG Biotech, Ebersberg, Germany) were used for obtaining DNA templates for *in vitro* transcription. The following GFP-specific primers were used: FSP6GFP: ATTTAGGTGACACTATAGAACCTGAAGTTCATCT GCACCA (T_m 60 °C); RT7GFP: TAATACGACTCACTATAGGGTGCT CAGGTAGTGGTTGTCG (T_m 60 °C). The expected size of the PCR product was 520 bp. For detection of lentiviral vector–derived LAMB3 RNA, the following primers were used: FSP6NVb3: ATTTAGGTGACACTAT AGAAGGACAGGATGAAAGACATGGA (T_m 60 °C) and RT7 NVb3: TAA TACGACTCACTATAGGGGGGCCACAACTCCTCATAAA (T_m 60 °C). The expected size of the PCR product was 540 bp. *In situ* RNA hybridization was performed as previously described.[33]

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