Regeneration of the entire human epidermis by transgenic stem cells

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Junctional Epidermolysis Bullosa (JEB) is a severe, often lethal genetic disease caused by mutations in genes encoding the basement membrane component laminin-332. Surviving JEB patients develop chronic skin and mucosa wounds, which impair their quality of life and lead to skin cancer. Here we show that autologous transgenic keratinocyte cultures regenerated an entire, fully functional epidermis on a 7-year-old child suffering from a devastating, life-threatening form of JEB. The proviral integration pattern was maintained *in vivo* and epidermal renewal did not cause any clonal selection. Clonal tracing showed that human epidermis is not sustained by equipotent progenitors, but by a limited number of long-lived stem cells, detected as holoclones, able to extensively self-renew *in vitro* and *in vivo* and to produce progenitors that replenish terminally differentiated keratinocytes. This study provides a blueprint that can be applied to other stem cell-mediated combined *ex vivo* cell and gene therapies.

Generalized Junctional Epidermolysis Bullosa (JEB) is a severe, often lethal genetic disease characterized by structural and mechanical fragility of the integuments. Skin and mucosal blisters and erosions occur within the lamina lucida of the basement membrane upon minor trauma. Massive chronic skin wounds greatly impair the patients' quality of life, lead to recurrent infections and scars and are predisposing to skin cancer. JEB is caused by mutations in *LAMA3*, *LAMB3* or *LAMC2* genes, which jointly encode laminin-332 (a heterotrimeric protein, also known as laminin 5, consisting of α , β , and γ chains) and in genes encoding collagen XVII and α β integrins[1]. Deleterious mutations causing absence of laminin-332 are usually early lethal. In nonlethal JEB, laminin-332 is strongly reduced and hemidesmosomes are rudimentary or absent. There is no cure for JEB and >40% of the patients succumb to the disease by adolescence[1,2]. Available symptomatic treatments can only relieve the devastating clinical manifestations.

Monthly renewal and timely repair of human epidermis is sustained by epidermal stem cells, which generate colonies known as holoclones[3,4]. Holoclones produce meroclone- and paraclone-forming cells, which behave as transient amplifying (TA) progenitors[3,4]. Epithelial cultures harbouring holoclone-forming cells can permanently restore massive skin and ocular defects[5-9]. A phase I/II clinical trial (1 patient) and a single-case study provided compelling evidence that local transplantation of transgenic epidermal cultures can generate a functional epidermis, leading to permanent (the longest follow-up being of

12 years) correction of JEB skin lesions[10-12]. However, paucity of treated areas (a total of ~0.06 m^2) did not significantly improve patients' quality of life[10-12].

A major criticism to this therapeutic approach has been its supposed unsuitability for the massive skin lesions marking generalized JEB. Here we show life-saving regeneration of virtually the entire epidermis (~0.85 m^2) on a 7-year-old child suffering from a devastating form of JEB by means of autologous transgenic keratinocyte cultures. The regenerated epidermis remained robust, resistant to mechanical stress and did not develop blisters or erosions during 21 months follow-up. Such fully functional epidermis is entirely sustained by a limited number of transgenic epidermal stem cells, detected as holoclones, able to extensively self-renew *in vitro* and *in vivo*.

The patient

In June 2015, a 7-year-old child was admitted to the Burn Unit of the Children's Hospital, Ruhr-University, Bochum, Germany. He carried a homozygous acceptor splice site mutation (C1977-1G > A, IVS 14-1G >A) within intron 14 of *LAMB3*. Since birth, the patient developed blisters all over his body, particularly on limbs, back and flanks. His condition severely deteriorated six weeks before admission, due to infection with *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Shortly after admission, he suffered complete epidermal loss on ~60% of the total body surface area (TBSA). During the following weeks, all therapeutic approaches failed and the patient's short-term prognosis was unfavourable (Methods). After the parents' informed consent, the regional regulatory authorities and the ethical review board of the Ruhr-University authorised the compassionate use of combined *ex vivo* cell and gene therapy. The parents of the patient also consented on the publication of the photographs and medical information included in this paper.

At the first surgery, the patient had complete epidermal loss on ~80% TBSA (Fig. 1a, b).

Regeneration of a functional epidermis by transgenic epidermal cultures

On September 2015, a 4-cm^2 biopsy, taken from a currently non-blistering area of patient's left inguinal region, was used to establish primary keratinocyte cultures, which were then transduced with a retroviral vector (RV) expressing the full-length *LAMB3* cDNA under the control of the Moloney leukaemia virus (MLV) long terminal repeat[13] (Methods, Extended Data Fig. 1 and Supplementary Information). Sequentially, 0.85 m^2 transgenic epidermal grafts, enough to cover all patient's denuded body surface, were applied on a properly

112 prepared dermal wound bed (Extended Data Fig. 2a). All limbs, flanks and the entire back
113 were grafted on October and November 2015. Some of the remaining denuded areas were
114 grafted on January 2016.

115 Previously, transgenic epidermal sheets were cultivated on plastic, enzymatically
116 detached from the vessel and mounted on a non-adhering gauze[10-12]. Keratinocyte
117 cultivation on a fibrin substrate – currently used to treat massive skin and ocular burns[6,8,9] -
118 eliminates cumbersome procedures for graft preparation and transplantation and avoids
119 epidermal shrinking, allowing the production of larger grafts using the same number of
120 clonogenic cells needed to produce plastic-cultured grafts. Since degradation of fibrin after
121 transplantation, which is critical to allow cell engraftment, was never assessed in a JEB
122 wound bed, at the first surgery we compared plastic- and fibrin-cultured grafts (Methods,
123 Extended Data Fig. 1).

124 The left arm received plastic-cultured grafts (Extended Data Fig. 2b, asterisks). Upon
125 removal of the non-adhering gauze (10 days post-grafting, Extended Data Fig. 2c, arrows),
126 epidermal engraftment was evident (asterisks). Epidermal regeneration, evaluated at 1
127 month, was stable and complete (Extended Data Fig. 2d). The left leg received both
128 plastic- and fibrin-cultured grafts (Extended Data Fig. 2e, asterisk and arrow, respectively),
129 both of which showed full engraftment at 10 days (Extended Data Fig. 2f, asterisk and
130 arrow, respectively) and complete epidermal regeneration at 1 month (Extended Data Fig.
131 2f, inset). Similar data were obtained on the other limbs. Thus, the patient's denuded back
132 (Extended Data Fig. 2g) received only fibrin-cultured grafts (inset). As shown in Extended
133 Data Fig. 2h, virtually complete epidermal regeneration was observed at 1 month, with the
134 exception of some areas (asterisks), some of which contained islands of newly formed
135 epidermis (arrows). Over the following weeks, the regenerated epidermis surrounding the
136 open lesions and those epidermal islands spread and covered most of the denuded areas
137 (Extended Data Fig. 2i). We then transplanted the remaining defects on flanks, thorax,
138 right thigh, right hand and shoulders. Epidermal regeneration was attained in most of those
139 areas.

140 Thus, ~80% of the patient's TBSA was restored by the transgenic epidermis (Fig. 1c).
141 During the 21 months follow-up (over 20 epidermal renewing cycles), the regenerated
142 epidermis firmly adhered to the underlying dermis, even after induced mechanical stress
143 (Fig. 1d and video in Supplementary Information), healed normally and did not form
144 blisters, also in areas where follow-up biopsies were taken (Fig. 1e, arrow).

The patient was discharged in February 2016 and is currently leading a normal social life. His epidermis is currently stable, robust, does not blister, itch, or require ointment or medications.

Ten punch biopsies were randomly taken, 4, 8 and 21 months after grafting. The epidermis had normal morphology and we could not detect blisters, erosions or epidermal detachment from the underlying dermis (Extended Data Fig. 3a). *In situ* hybridization using a vector specific *t-LAMB3* probe showed that the regenerated epidermis consisted only of transgenic keratinocytes (Fig. 2a). At admission, laminin 332-β was barely detectable in patient's skin (Fig. 2b). In contrast, control and transgenic epidermis expressed virtually identical amounts of laminin 332-β, which was properly located at the epidermal-dermal junction (Fig. 2b). The basal lamina contained normal amounts of laminin 332-α3 and γ2 chains and α6β4 integrins, all of which were strongly decreased at admission (Extended Data Fig. 3b). Thus, transduced keratinocytes restored a proper adhesion machinery (Extended Data Fig. 3c). Indeed, the transgenic epidermis revealed normal thickness and continuity of the basement membrane (Fig. 2c, arrowheads) and normal morphology of hemidesmosomes (Fig. 2c, arrows). At 21 months follow-up, the patient's serum did not contain autoantibodies directed against the basement-membrane zone (Extended Data Fig. 3d).

In summary, transgenic epidermal cultures generated an entire functional epidermis in a JEB patient. This is consistent with the notion that keratinocyte cultures have been used for decades to successfully treat life-threatened burn victims on up to 98% of TBSA[5,6,9,14]. It can be argued that the patient's clinical picture (massive epidermal loss, critical conditions, poor short-term prognosis) was unusual and our aggressive surgery (mandatory for this patient) unthinkable for the clinical course of most EB patients. But progressive replacement of diseased epidermis can be attained in multiple, less invasive surgical interventions on more limited body areas. EB has the advantage of a preserved dermis (not available in deep burns), which allows good functional and cosmetic outcomes. This approach would be optimal for newly diagnosed patients early in their childhood. A bank of transduced epidermal stem cells taken at birth could be used to treat skin lesions while they develop, thus preventing, rather than restoring, the devastating clinical manifestations rising through adulthood. Currently, combined *ex vivo* cell and gene therapy cannot be applied to lesions of the internal mucosae, which, however, are usually more manageable than those on skin, perhaps with the exception of oesophageal strictures.

179

180 **Integration profile of transgenic epidermis**

181 Pre-graft transgenic cultures (PGc) were generated by ~$8.7x10^6$ primary clonogenic cells
182 and consisted of $2.2x10^8$ keratinocytes (divided in 36 vials), ~45% of which were seeded to
183 prepare 0.85 m^2 transgenic epidermal grafts (Extended Data Fig. 1).

184 To investigate the genome-wide integration profile, 3 PGc samples were sequenced
185 using two independent LTR-primers (i.e., 3pIN and 3pOUT, Supplementary Table 1) for
186 library enrichment (n=12; see Methods). High-throughput sequencing recovered a total of
187 174.9M read pairs and the libraries obtained using the two LTR-primers showed similar
188 number of reads and comparable insertion counts (Pearson R>0.92, p<0.005). After
189 merging all integration sites from the two independent priming systems, we identified
190 27,303 integrations in PGc (Fig. 3a, bars) with an average coverage of 2.5 reads/insertion
191 (Fig. 3a, lines and Supplementary Table 4). The same analysis was performed on primary
192 cultures initiated from 3 biopsies (~0.5 cm^2 each) taken at 4 (left leg) and 8 (left arm and
193 left leg) months after grafting, referred to as 4Mc, $8Mc_1$, and $8Mc_2$, respectively (Methods).

194 Strikingly, we detected only 400, 206, and 413 integrations in 4Mc, $8Mc_1$, and $8Mc_2$,
195 respectively (Fig. 3a, bars) with an average coverage of 27.3, 19.5, and 20.4 (Fig. 3a,
196 lines).

197 To exclude that the major difference in the number of integrations found in pre- and
198 post-graft samples could be ascribable to PCR reactions causing unbalanced
199 representation of event-specific amplicons, or to spatiality-effect of punch biopsies, we
200 estimated the expected number of PGc, 4Mc, $8Mc_1$, and $8Mc_2$ integrations using the
201 Chapman-Wilson capture-recapture model on the data obtained from the independent
202 libraries (Methods)[15]. In PGc, the model estimated 65,030±2,120 integrations, *i.e.*
203 approximately twice the actual number of detected insertions. The same model estimated
204 457±31, 323±50, and 457±24, independent integrations in 4Mc, $8Mc_1$, and $8Mc_2$,
205 respectively (confidence level of 99%, α=0.01), which is highly consistent with the number
206 of events actually detected. Of note, 58%, 43% and 37% of 4Mc, 8Mc1 and 8Mc2
207 integrations, respectively, were identified in PGc (Fig. 3b), which is consistent with the
208 percentage (~50%) of insertions detected in PGc by NGS analysis.

209 Integrations were mapped to promoters (defined as 5 kb regions upstream the
210 transcription start site of RefSeq genes), exons, introns, and intergenic regions. In all pre-
211 and post-graft samples, ~10% of events were located within promoters. The majority of
212 integrations were either intronic (~47%) or intergenic (~38%) and less than 5% were found

in exons (Fig. 3c, left panel). We also annotated integrations in epigenetically defined transcriptional regulatory elements (Methods and Supplementary Information). As shown in Fig. 3c (right panel), ~27% of integrations were associated to active promoters or enhancers and no significant difference in the distribution of insertions was detected in pre- and post-graft samples (p-value>0.05; Pearson's Chi-squared test). Thus, the integration pattern was maintained *in vivo* and epidermal renewal did not determine any clonal selection.

Genes containing an integration were not functionally enriched in Gene Ontology categories related to cancer-associated biological processes[16], with the exception of cell migration and small GTPase mediated signal transduction (Fig. 3d and Extended Data Table 1a). These findings are however expected, since our culture conditions are optimized to foster keratinocyte proliferation and migration, to sustain clonogenic cells and to avoid premature clonal conversion and terminal differentiation, all of which are instrumental for the proper clinical performance of cultured epidermal grafts[14]. Thus, similarly to what has been reported in transgenic hematopoietic stem cells[17,18], our high-throughput analyses revealed a cell-specific vector preference that is related to the host cell status in terms of chromatin state and transcriptional activity at the time of transduction[19].

MLV-RV vectors raised concerns about insertional genotoxicity, which has been reported with hematopoietic stem cells, but in specific disease contexts[17,20-22]. Indeed, a γRV vector, similar to ours, obtained a marketing authorization for *ex vivo* gene therapy of adenosine deaminase severe combined immunodeficiency and has been approved for PhaseI/II clinical trials on RDEB (https://clinicaltrials.gov/ct2/show/NCT02984085)[23]. The patient's integration profile confirmed absence of clonal selection both *in vitro* and *in vivo*. Likewise, we never observed immortalization events related to specific proviral integrations in many serially cultivated MLV-RV-transduced keratinocytes. Two JEB patients, receiving a total of ~$1x10^7$ clonogenic transgenic keratinocytes in selected body sites (3.5 and 12 years follow-up)[10-12], and the patient, receiving ~$3.9x10^8$ transgenic clonogenic cells all over his body (Extended Data Fig. 1), did not manifest tumour development or other related adverse events. Therefore, based on *in vivo* data, the frequency of a detectable transformation event (if any) in MLV-RV-transduced keratinocytes would be less than 1 out of $1x10^7$ during the first 12 years follow-up. Although the follow up of this patient is shorter and does not allow drawing definitive conclusions, the frequency of detectable insertional mutagenesis events to date is less than 1 out of $3.9x10^8$. In evaluating the risk/benefit

ratio, it should also be considered that severely affected JEB patients are likely to develop aggressive squamous cell carcinoma as a consequence of the progression of the disease.

The transgenic epidermis is sustained by self-renewing stem cells (holoclones).

The percentage of clonogenic cells, including holoclones, remained relatively constant during the massive cell expansion needed to produce the grafts (Extended Data Fig. 1 and Extended Data Table 2). The patient received ~3.9×10^8 clonogenic cells, ~1.6×10^7 of which were holoclone-forming cells, to cover ~0.85 m^2 of his body (Extended Data Fig. 1 and 4 and Extended Data Table 2). Thus, ~$4.6 \times 10^4/cm^2$ clonogenic cells or ~$1.8 \times 10^3/cm^2$ stem cells were transplanted on the patient's body surface (Extended Data Fig. 4).

If originally transduced clonogenic cells were all long-lived equipotent progenitors, (*i*) we would have recovered thousands of integrations per cm^2 of regenerated epidermis; (*ii*) all clonogenic cells contained in 4Mc, $8Mc_1$ and $8Mc_2$ cultures would have independent integrations, irrespectively of the clonal type. Instead, if the transgenic epidermis was sustained only by a restricted number of long-lived stem cells (continuously generating pools of TA progenitors), (*i*) we would have recovered, at most, only few hundreds of integrations per cm^2; (*ii*) mero- and paraclones contained in 4Mc, $8Mc_1$ and $8Mc_2$ cultures would have the same integrations found in the corresponding holoclones.

The number of integrations detected in post-graft cultures (Fig. 3a) is consistent with the number of stem cells that have been transplanted (Extended Data Fig. 4), hence it strongly supports the latter hypothesis, which was verified by proviral analyses at clonal level (Extended Data Fig. 5) on PGc, 4Mc and $8Mc_1$. A total of 687 clones (41 holoclones and 646 mero/paraclones) were analysed. PGc, 4Mc and $8Mc_1$ generated 20, 14 and 7 holoclones and 259, 264 and 123 mero/paraclones, respectively. Thus, PGc, 4Mc and $8Mc_1$ contained 7.2%, 5.0% and 5.4% holoclone-forming cells, respectively (Extended Data Table 2). Each clone was cultivated for further analysis. Libraries of vector-genome junctions, generated by linear-amplification-mediated (LAM) PCR followed by pyrosequencing, retrieved 31 independent integrations unambiguously mapped on the genome of holoclones (Extended Data Table 1b). One holoclone (4Mc) was untransduced, 28, 11 and 1 holoclones contained 1, 2 and 3 integrations, respectively. Eleven holoclones in 4Mc shared the same integration pattern. The same happened for two couples of holoclones in $8Mc_1$. Holoclones' copy numbers were confirmed by RTq-PCR (Extended Data Fig. 6). Strikingly, 75% and 80% of integrations found in 4Mc and $8Mc_1$ holoclones were retrieved in PGc, respectively (Fig. 4a), supporting the NGS-based survey as well as

a representative sampling. The integration pattern observed in holoclones confirms absence of selection of specific integrations during epidermal renewal *in vivo* (Fig. 4b) and mirrors the pattern found in their parental cultures (Fig. 3c), including absence of genes associated to cell cycle control, cell death, or oncogenesis (Fig. 3d and Extended Data Table 1a).

Clonal tracing was then performed by PCR, using genomic coordinates of holoclone insertions. As expected, the vast majority of PGc meroclones and paraclones (91%) did not contain the same integrations detected in the corresponding holoclones (Fig. 4c, PGc). Such percentage decreased to 37% already at 4 months after grafting (Fig. 4c, 4Mc). Strikingly, virtually the entire clonogenic population of primary keratinocyte cultures established at 8 months contained the same integrations detected in the corresponding holoclones (Fig 4c, $8Mc_1$). Thus, the *in vivo* half-live of TA progenitors is of approximately 3-4 months. These data formally show that the regenerated epidermis is sustained only by long-lived stem cells (holoclones) and underpins the notion that meroclones and paraclones are short-lived progenitors continuously generated by the holoclones, both *in vitro* and *in vivo*. The high percentage of holoclone integrations retrieved in PGc, together with the number of shared events across cultures (Fig. 3b), suggests that the average coverage of the NGS analysis in PGc allowed to preferentially identify integrations in holoclones and in TA cells deriving from such holoclones already during the cultivation process.

In summary, as depicted in Extended Data Fig. 7, altogether these findings demonstrate that (*i*) PGc consisted of a mixture of independent transgenic holoclones, meroclones and paraclones, (*ii*) meroclones and paraclones (which can be isolated directly from a skin biopsy) are TA progenitors, do not self-renew and are progressively lost during cultivation and *in vivo* epidermal renewal, hence do not contribute to long-term maintenance of the epidermis; (*iii*) the transgenic epidermis is sustained only by long-lived stem cells detected as holoclones; (*iv*) founder stem cells contained in the original primary culture must have gone extensive self-renewal (*in vitro* and *in vivo*) to ultimately sustain the regenerated epidermis, as confirmed by the number of shared events across samples and across holoclones.

DISCUSSION

The entire epidermis of a JEB patient can be replaced by autologous transgenic epidermal cultures harbouring an appropriate number of stem cells. Both stem and TA progenitors are instrumental for proper tissue regeneration in mammals[24]. However, the nature and the

properties of mammalian epidermal stem cells and TA progenitors are a matter of debate[25,26]. Although epidermal cultures have been used for 30 years in the clinic[14], a formal proof of the engraftment of cultured stem cells has been difficult to obtain. Similarly, the identification of holoclones as human epithelial stem cells and mero/paraclones as TA progenitors and their role in long-term human epithelial regeneration have been inferred from compelling, yet indirect evidence[6,8,9,27]. Using integrations as clonal genetic marks, we show that the vast majority of TA progenitors are progressively lost within a few months after grafting and the regenerated epidermis is indeed sustained only by a limited number of long-lived, self-renewing stem cells. Similar data have been produced with transgenic hematopoietic stem cells[28]. This notion argues against a model positing the existence of a population of equipotent epidermal progenitors that directly generate differentiated cells during the lifetime of the animal[25] and fosters a model where specific stem cells persist during the lifetime of the human and contribute to both renewal and repair by giving rise to pools of progenitors that persist for various periods of time, replenish differentiated cells and make short-term contribution to wound healing[26]. Hence, the essential feature of any cultured epithelial grafts is the presence (and preservation) of an adequate number of holoclone-forming cells. The notion that the transgenic epidermis is sustained only by engrafted stem cells further decreases the potential risk of insertional oncogenesis.

In conclusion, transgenic epidermal stem cells can regenerate a fully functional epidermis virtually indistinguishable from a normal epidermis, so far in the absence of related adverse events. The different forms of EB affect approximately 500,000 people worldwide (http://www.debra.org). The successful outcome of this study paves the road to gene therapy of other types of EB and provides a blueprint that can be applied to other stem cell-mediated combined *ex vivo* cell and gene therapies.

Methods

Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper. All data used to generate main and supplementary figures are provided as source data files.

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421

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441

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443 defined strategic procedures, performed transplantation of the transgenic grafts, surgical
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446 figures and edited the manuscript, D.S., I.J., M.M. performed integration profile of
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451 M, K carried out the follow-up of patient, D.Q. performed electron microscopy analysis;
452 M.D.L. coordinated the study, defined strategic procedures, administered the experiments
453 and wrote the manuscript. Correspondence and requests for materials should be
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455
456 **Competing financial interests** G.P. and M.D.L. are co-founders and member of the
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458 Farmaceutici S.p.A. (a co-founder of HTA), holds an Orphan Drug Medicinal Product
459 designation (EU/3/15/1465) for the transgenic cultures used in this paper.
460
461 **Figure Legends**
462
463 **Figure 1. Regeneration of the transgenic epidermis.**
464 **a**, Clinical picture of the patient showing massive epidermal loss. **b**, Schematic
465 representation of the clinical picture. The denuded skin is indicated in red, while blistering
466 areas are indicated in green. Flesh-colored areas indicate currently non-blistering skin.
467 Transgenic grafts were applied on both red and green areas. **c**, Restoration of patient's
468 entire epidermis, with the exception of very few areas on the right thigh, buttocks, upper
469 shoulders/neck and left axilla (asterisks, altogether ≤2% of TBSA). **d**, Normal skin
470 functionality and elasticity. **e**, Absence of blister formation at sites where some of post-
471 graft biopsies were taken (arrow).
472
473 **Figure 2. Restoration of a normal epidermal-dermal junction.**
474 Skin sections were prepared from normal skin, patient' affected (admission) and
475 transgenic skin at 4, 8 and 21 months follow-up. **a**, *In situ* hybridization was performed
476 using a transgene-specific probe (*t-LAMB3*) on 10-μm-thick sections. E-cadherin-specific
477 probe (*Cdh1*) was used as a control. Scale bars, 40 μm. **b**, Immunofluorescence of laminin
478 332-β3 was performed with 6F12 moAbs on 7-μm-thick sections. DAPI (blue) marks
479 nuclei. Dotted line marks the epidermal-dermal junction. Scale bars, 20 μm. **c**, Electron-

480 microscopy was performed on 70-nm-thick skin sections. A regular basement membrane
481 (arrows) and normal hemidesmosomes (arrowheads, higher magnification in the inset) are
482 evident in patient' transgenic skin. Scale bars, 1 μm.
483
484 **Figure 3. Integration profile of transgenic epidermis.**
485 **a**, Integrations were identified in libraries obtained using two LTR-primers (3pIN, light grey
486 bars; 3pOUT, dark grey bars; Supplementary Table 1) and in the merged set (black bars).
487 Lines (secondary axis) depict the average integration coverage, calculated after removal of
488 PCR duplicates. **b**, Venn diagram of the number of shared integrations across samples. **c,**
489 percentage of integrations mapped to: promoters, exons, introns, and intergenic regions
490 (left); epigenetically defined active and weak promoters and enhancers, or genomic
491 regions with no histone marks (right); (p-value>0.05; Pearson's Chi-squared test). **d**, Dot
492 plot of the top 5 enriched GO Biological Process terms for each sample. Dot colour
493 indicates statistical significance of the enrichment (q-value); dot size represents the
494 fraction of genes annotated to each term.
495
496 **Figure 4. Integration profile of stem and TA cells.**
497 **a**, Percentage of holoclone integrations recovered in the PGc bulk population. **b**,
498 Holoclone integrations mapped to: promoters, exons, and introns, and intergenic regions
499 (left); epigenetically defined active and weak promoters and enhancers, or genomic
500 regions with no histone marks (right). **c**, The PGc pie chart (grey segment) shows that
501 91% of mero/paraclones did not contain the same integrations detected in the
502 corresponding holoclones (each indicated by different blue segments). The 4Mc and $8Mc_1$
503 pie charts (grey segments) show that such percentage decreased to 37% and 13%,
504 respectively.
505
506 **EXTENDED DATA FIGURE LEGENDS**
507
508 **Extended Data Figure 1. Schematic representation of combined *ex vivo* cell and**
509 **gene therapy.**
510 The scheme shows the entire procedure, from skin biopsy to transplantation and follow up.
511 Total number of keratinocytes, the corresponding clonogenic fraction and days of
512 cultivation are shown for each passage. All analyses performed at each follow-up are
513 indicated. Immunofluorescence (IF), *in situ* hybridization (ISH) and transmission electron

514 microscopy (TEM) were performed on randomly taken 0.2-0.4 mm^2 punch biopsies.
515 Genome-wide analysis (NGS) was performed on Pre-Graft cultures (PGc) and on primary
516 cultures initiated from ~0.5 cm^2 biopsies taken from the left leg (4Mc and 8Mc2) and the
517 right arm (8Mc1). Clonal analysis and tracing were performed on PGc, 4Mc and 8Mc1
518

519 **Extended Data Figure 2. Regeneration of the epidermis by transduced keratinocyte**
520 **cultures.**
521 **a**, Preparation of a dermal wound bed at the time of transplantation. **b**, Transplantation on
522 the left arm of plastic-cultured epidermal grafts, mounted on a non-adhering gauze
523 (asterisks). **c**, The engrafted epidermis (asterisks) is evident upon removal of the gauze
524 (arrows), 10 days after grafting. **d**, Regenerated epidermis on the left arm at 1 month. **e**,**f**,
525 Transplantation (**e**) and engraftment (**f**) of both plastic-cultured (asterisk) and fibrin-
526 cultured (arrow and inset in **e**) grafts on the left leg. **f** (inset), Complete epidermal
527 regeneration is evident at 1 month. **g**, The back of the patient was covered by fibrin-
528 cultured grafts (inset). **h**, Complete epidermal regeneration was observed at 1 month, with
529 the exception of some areas marked by the asterisks. Islands of epidermis were observed
530 inside those denuded areas (arrows). **i**, Within 4 months, the regenerated epidermis
531 surrounding the open lesions and the epidermal islands detected within those open lesions
532 spread and covered the denuded areas.
533

534 **Extended Data Figure 3. Restoration of a normal dermal-epidermal junction.**
535 **a**, Hematoxylin/Eosin staining of skin sections (7-μ -thick) prepared from normal skin and
536 from the patient at admission and at 4, 8 and 21-months follow-up. Black arrows show
537 ruptures at the epidermal-dermal junction. Scale bar, 20 μm. **b**, Sections (7-μ -thick) from
538 normal skin, the patient's skin at admission and 21 months after transplantation were
539 immunostained using laminin 332-α3, laminin 332-γ2, α6 integrin and β4 integrin
540 antibodies. **c**, Adhesion of cohesive cultured epidermal sheets. Upper panel: spontaneous
541 detachment (arrow) of confluent laminin 332-β3 null patient's keratinocyte cultures. Lower
542 panel: genetically corrected patient's cultures remained firmly attached to the substrate. As
543 with normal control cells, their detachment would have required prolonged enzymatic
544 treatment. **d**, The absence of a humoral immune response to the transgene product was
545 verified by indirect immunofluorescence performed on monkey esophagus and normal
546 human split skin (NH-SS) sections, using the patient's plasma taken 21 months after
547 transplantation. An anti-human laminin-332 antibody (anti-GB3) was used as positive

548 control (C+). A healthy donor's plasma was used as negative control (C-). Arrows denote
549 the expected localization of the laminin-332 labelling. Scale bar 100 μm.
550
551 **Extended Data Figure 4. Schematic model of epidermal stem/progenitor function.**
552 Clonogenic progenitors (blue cells) contained the original skin biopsy and in 8,472 cm^2 of
553 transgenic epidermis are indicated. Stem cells, detected as holoclones (pink cells), were
554 identified by clonal analysis (Methods and Extended Data Fig. 5). The number of
555 holoclones contained in the primary culture has been estimated. The schematic model
556 posits the existence of specific long-lived stem cells generating pools of short-lived
557 progenitors (Hypothesis 1) or a population of equipotent epidermal progenitors
558 (Hypothesis 2). The number of integrations predicted by the Chapman-Wilson capture and
559 re-capture model and formally detected by NGS analysis in 4Mc, 8Mc1 and 8Mc2 (right
560 part of the panel) is consistent with the number of transplanted holoclones, hence fosters
561 Hypothesis 1.
562
563 **Extended Data Figure 5. Clonal analysis scheme.**
564 Sub-confluent cultures were trypsinized, serially diluted and inoculated (0.5 cell/well) onto
565 96-multiwell plates containing irradiated 3T3-J2 cells. After 7 d of cultivation, single clones
566 were identified under an inverted microscope (Scale bar, 100μm), trypsinized, transferred
567 to 2 dishes and cultivated. One dish (1/4 of the clone) was fixed 12 d later and stained with
568 Rhodamine B for the classification of clonal type. The clonal type was determined by the
569 percentage of aborted colonies formed by the progeny of the founding cell. The clone was
570 scored as holoclone when 0–5% of colonies were terminal. When 95-100% of colonies
571 were terminal (or when no colonies formed), the clone was classified as paraclone. When
572 the amount of terminal colonies was between 5% and 95%, the clone was classified as
573 meroclone. The second dish (3/4 of the clone) was used for integration analysis after 7 d
574 of cultivation.
575
576 **Extended Data Figure 6. Determination of provirus copy number.**
577 Quantitative PCR (qPCR) was performed on genomic DNA of pre-graft cultures (PGc),
578 primary cultures generated at 4 months (4Mc) and 8 months (8Mc1, 8Mc2) follow-up and
579 selected holoclones (PRE.G_H1, PRE.G_H10, FU4m_H1-11, PRE.G_H7). All values are
580 represented as the mean of 2 independent qPCR ± SEM.
581

582 **Extended Data Figure 7. Schematic model of holoclone tracing in the regenerated**
583 **patient's epidermis.**
584 Transgenic epidermal cultures (PGc) contain of a mixed population of clonogenic basal
585 stem cells (blue) and TA progenitors (grey). Upon engraftment and initial epidermal
586 regeneration, both stem and TA cells can proliferate and eventually generate suprabasal
587 terminally differentiated cells. Upon epidermal renewal (4 and 8 months), the short-lived
588 TA progenitors (grey) are progressively lost. The long-lived stem cells then generate new
589 pools of TA progenitors (now blue basal cells), which will produce terminally differentiated
590 cells (suprabasal blue cells).
591
592 **Extended Data Figure 8. Clinical data.**
593 During hospitalization, the patient's inflammatory and nutritional status was documented
594 by blood concentration of **a**, C-reactive protein (CRP) and **b**, albumin. The time course of
595 biopsy sampling (marked by "B") and epidermal culture transplantation is given by the
596 arrows. The linear regressions visualize the trend of pre graft (dotted) and post graft (black
597 line) progressions. The red line within the CRP time course demonstrates the CRP-limit,
598 which is considered as a criterion for severe inflammation. These data demonstrate the
599 critical situation of the patient at admission and before transplantation and the
600 improvement of his general status upon epidermal regeneration.
601
602 **Extended Data Table 1. a.** Enrichment of cancer-related biological process in genes
603 harboring an insertion. Statistical significant enrichments at a 95% confidence level (q-
604 value ≤ 0.05 in a Fisher's exact test) are in bold. GO categories were selected to represent
605 the cancer hallmarks described in Hanahan D, Weinberg RA. Cell. 2011 Mar 4;144(5):646-
606 74. **b.** Genomic and functional annotations of integrations in holoclones.
607
608 **Extended Data Table 2.** Clonal analysis was performed on pre-graft cultures (PGc), a
609 graft ready for transplantation (Graft) and on primary cultures established at 4 (4Mc) and 8
610 (8Mc1) months after grafting. H, M and P indicate holoclones, meroclones and paraclones,
611 respectively. Frequency indicates the percentage of holoclones detected.in the population
612 of clonogenic keratinocytes. Graft was not used for LAM-PCR or NGS analyses but for
613 holoclone quantification as part of quality control of the process.
614
615 **METHODS**

616

617 **Ethics statement**

618 Five weeks after the patient's admission, we considered a palliative treatment, as the
619 clinical situation had deteriorated. The patient's father asked for possible experimental
620 treatments. We informed the parents on the possibility of the transplantation of genetically
621 modified epidermal cultures. With the help of an interpreter, the parents were informed that
622 the aforementioned procedure had been applied only on two patients with epidermolysis
623 bullosa and on limited body sites. They were also informed that, given the patient's critical
624 conditions, the complexity of the entire surgical procedure needed for graft application
625 could have been itself lethal. The potential risk of tumour development within the
626 transplant was also discussed. As the parents still expressed their wish to use this
627 experimental procedure, the local research ethics committee of the Medical Faculty of the
628 Ruhr-University Bochum, contacted in July 2015, gave its approval to perform the
629 procedure if responsible authorities approved the proposed treatment in our patient. We
630 contacted the Paul-Ehrlich-Institut, which referred the request to the District Council of
631 Arnsberg. The District Council of Arnsberg, North Rhine-Westphalia, Germany, which was
632 responsible for the approval of committed treatments with new medical products,
633 authorized the compassionate use of combined *ex vivo* cell and gene therapy in August
634 2015. The District Council of Duesseldorf, North Rhine-Westphalia, Germany, approved
635 the genetic engineering work according to the Act on Genetic Engineering §9 Abs. 2
636 GenTG on the basis of the pre-existing approval for the Gene Technology Lab Security
637 Level 2, which had been amended to the operating room of the BG University Hospital
638 Bergmannsheil, Ruhr-University Bochum in August 2015.

639 The entire procedure used to prepare the transgenic epidermis has been previously
640 scientifically reviewed and evaluated by the Italian Ministry of Health and approved by the
641 ethical review board of the University of Modena and Reggio Emilia, both of which
642 approved a phase I/II clinical trial with the very same transgenic cultures in June 2015.
643 Similarly, the Austrian regulatory authorities scientifically reviewed and approved 2
644 additional clinical trials envisaging the use of very similar transgenic cultures, the only
645 difference being the transgene used in the vector.

646 All procedures were performed in adherence to the last available (2008) version of the
647 International Society for Stem Cell Research (ISSCR) "Guidelines for the Clinical
648 Translation of Stem Cells". Since all legal requirements currently required in Germany to
649 obtain the approval for the treatment were fully met and the clinical condition of the patient

650 was rapidly deteriorating, we opted to proceed with the life saving treatment, which was
651 started in September 2015, after obtaining the parents' informed consent. All documents
652 were presented to the parents in German and their native language translated by an
653 accredited translator. The patient's parents also consented on the publication of
654 photographs and medical information included in this publication. All photographs were
655 presented to them before signing the consent forms.
656

657 **Patient, clinical course, surgical, and post-operative procedures.**

658 Since birth, the patient repeatedly developed blisters, upon minor trauma, on the back, the
659 limbs and the flanks, which occasionally caused chronic wounds persisting up to one year.
660 Six weeks before the actual exacerbation, his condition deteriorated with the development
661 of massive skin lesions. One day prior to admission, he developed fever followed by
662 massive epidermal loss. He was admitted to a tertiary care hospital where topical wound
663 care was performed using absorbable foam dressings (Mepilex, Mölnlycke Healthcare,
664 Erkrath, Germany). As the patient appeared septic with elevated infection parameters, he
665 initiated systemic antibiotic treatment with meropenem and vancomycin. Severe electrolyte
666 imbalances required parenteral substitution of sodium, potassium, and magnesium. Swabs
667 revealed *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Due to the large wound
668 area and further deterioration of his clinical condition, the patient was transferred to the
669 paediatric burn centre of the Ruhr-University 4 days later. At admission, he suffered
670 complete epidermal loss on ~60% of total body surface area (TBSA), affecting all limbs,
671 the back and the flanks. The patient was febrile, cachectic, with a total body weight of 17
672 kg (below 3rd percentile), had signs of poor perfusion and C-reactive protein (CRP) was
673 150 mg/L. Antibiotic treatment was continued according to microbiologic assessment with
674 flucloxacilline and ceftazidime. Retrospectively, the diagnosis of staphylococcal scalded
675 skin syndrome was suspected due to flaky desquamations appearing 10 d after the
676 symptoms began and *Staphylococcus aureus* was found on swabs. The iscorEB clinician
677 score[29] was rated at 47. We initiated aggressive nutritional therapy by nasogastric tube
678 (1100-1300 kcal/d) and additional parenteral nutrition (700 kcal/d kcal/kg/d, glucose 4
679 g/kg/d, amino acids 3 g/kg/d, fat 1.5 g/kg/d) according to his nutritional demands
680 calculated using the Galveston formula. A necessary intake of about 1800 kcal/d was
681 determined. Vitamins and trace elements were substituted as needed since zinc,
682 selenium, and other trace elements were below the detection threshold. Beta-adrenergic
683 blockade with propranolol was also started, as with severe burns[30]. Due to bleeding during

684 dressing changes and on-going loss of body fluids from the widespread skin erosions, the
685 transfusion of 300 ml packed red blood cells was required every 7 to 12 days to keep the
686 Hb value above 6-7 g/dl, and 20 g albumin were substituted once per week to keep
687 albumin levels above 2.0 g/dl. Patient care was performed in accordance with the
688 epidermolysis bullosa treatment guidelines[31]. The patient was bathed in povidone-iodine
689 (PVP) solution or rinsed with polyhexanide-biguanide solution (PHMB) under general
690 anaesthesia, first on a daily basis and subsequently every other day. We also employed
691 several topical wound dressings and topic antimicrobials, including PHMB-gel and PVP
692 ointment, without any significant impact on wound healing. However, wounds became
693 cleaner and *Staphylococcus aureus* were no longer detectable for several weeks. The
694 patient had persistent systemic inflammatory response syndrome (SIRS) with spiking
695 fevers, wasting, and high values of acute-phase proteins (CRP, ferritin). He had chronic
696 pain necessitating comprehensive drug management using fentanyl, dronabinol,
697 gabapentin, amitryptiline and NSAIDs. Antibiotic treatment was continued according to
698 swabs taken once weekly; swabs revealed intermittent wound infection with *Pseudomonas*
699 *aeruginosa* and in the course *Enterobacter cloacae*, *Enterococcus faecalis* and again
700 *Staphylococcus aureus*. Treatment was changed biweekly omitting glycopeptides,
701 carbapenemes and other drugs of last resort using mainly ceftazidime, cefepime,
702 ampicilline, flucloxacilline, and tobramycin. Due to his life-threatening condition, we
703 performed an unsuccessful allotransplantation of split-thickness skin grafts taken from his
704 father. Despite an initial engraftment, complete graft loss occurred 14 days post-
705 transplantation. Treatment attempts with Suprathel (Polymedics Innovation GmbH,
706 Denkendorf, Germany), amnion, and glycerol preserved donor skin (Glyaderm, Euro
707 Tissue Bank, Beverwijk, Netherlands) were unsuccessful as well. Further treatment
708 attempts were judged to be futile by several experts in this field. After 5 weeks at the
709 intensive care unit, the patient no longer tolerated nutrition via nasogastric or duodenal
710 tube and began to vomit after small amounts of food. Due to massive
711 hepatosplenomegaly, a PEG or PEJ was not feasible. A Broviac catheter was implanted
712 and total parenteral nutrition was begun (1500 kcal/d, glucose 14 g/kg/d, amino acids 4
713 g/kg/d, fat 2 g/kg/d). Following an attempt of increased fat administration via parenteral
714 nutrition, the patient developed a pancreatitis that resolved after omitting fat from the
715 parenteral nutrition for a few days. With this nutritional regimen the patient's weight
716 remained stable and blood glucose below 150 mg/dl was obtained without insulin
717 administration. At this point, palliative care seemed the only remaining option. Because of

718 the very poor short-term prognosis, we decided to start an experimental therapy approach
719 using autologous epidermal stem cell-mediated combined *ex-vivo* cell and gene therapy
720 (see Ethics Statement). Transgenic grafts were prepared, free of charge, under Good
721 Manufacturing Practices (GMP) standards by Holostem Terapie Avanzate S.r.l. at the the
722 Centre for Regenerative Medicine "*Stefano Ferrari*", University of Modena and Reggio
723 Emilia, Modena, Italy. On October 2015, we performed the first transplantation of
724 transgenic cultures on the 4 limbs (and part of the flanks). At that time, the patient suffered
725 complete epidermal loss on ~80% of his body and still needed transfusion of 300 ml
726 packed red blood cells every 7 to 12 days and 20 g albumin once per week to keep the
727 albumin level above 2.0 g/dl. He continued suffering from spiking fevers, wasting, and high
728 values for acute-phase proteins (CRP, Ferritin). Wounds were colonized with
729 *Staphylococcus aureus* and *Escherichia coli*. Perioperative antibiotic therapy was
730 performed with flucloxacilline, ceftazidime and ciprofloxacine. Under general anaesthesia,
731 a careful and thorough disinfection with octenidine dihydrochloride (Schuelke & Mayr,
732 Norderstedt, Germany) and surgical debridement of all limbs and flanks was performed,
733 both with copper sponges and surgical knife. The debrided areas demonstrated a good
734 perfusion with intact dermis. After achieving haemostasis using epinephrine soaked gauze,
735 all debrided areas were washed thoroughly with saline to prevent epinephrine contact with
736 cultured grafts. Grafts were carefully transplanted on the denuded, debrided areas and
737 covered with Adaptic, a non-adhering dressing (Systagenix Wound Management,
738 Gargrave, UK) and sterile dressing. Post-operatively, as total immobilization was
739 recommended after the transplantation, the patient was maintained under continuous
740 isoflurane sedation for 12 days using the AnaConDa system (SedanaMedical, Uppsala,
741 Sweden). A catheter related blood-stream infection was successfully treated with
742 vancomycin and meropeneme. Despite the use of clonidine and propofol, the patient
743 developed a severe delirium after the isoflurane sedation, which was solved by
744 levomepromazine. Engraftment was evaluated at 8-14 days. Epidermal regeneration was
745 evaluated at 1 month (see text). Following the first transplantation, regular weekly
746 transfusion of red blood cells and infusion of albumin was no longer necessary. The
747 general condition improved and enteral nutrition became feasible again with the patient
748 tolerating up to 400 kcal/d via nasogastric tube complementing the parenteral nutrition
749 (1500 kcal/d, glucose 14 g/kg/d, amino acids 4 g/kg/d, fat 2 g/kg/d)[32]. On November 2015,
750 a second transplantation was performed on the dorsum, the buttocks (and small areas on
751 the shoulders and the left hand). These wounds were colonized with *Staphyloccus*

752 *epidermidis* and *Enterococcus faecium* at the time of transplantation. Antibiotic treatment
753 was done with vancomycin and ceftazidime due to suspected infection of the Broviac
754 catheter. However, due to the high risk and severe side effects of long-term sedation, the
755 patient was not sedated after the second transplantation. All dressings at the back and the
756 buttocks had to be removed due to infection with *enterococcus faecium* four days after
757 transplantation. Topical antimicrobial therapy using polihexanide was started. On the
758 dorsum, the graft healed in the following four weeks despite the early infection, and a
759 stable skin without blister formation appeared (see text). Four weeks after the second
760 transplantation, the CRP values remained below 100 mg/L and the patient was no longer
761 febrile (Extended Data Fig. 8). Complete enteral nutrition became feasible again. The
762 affected body surface area remained below 10% TBSA. On January 2016, we performed a
763 third procedure in a similar fashion covering the remaining defects on flanks, thorax, right
764 thigh, right hand, and shoulders. These wounds were colonized with *Staphylococcus*
765 *epidermidis*. The transplanted cells engrafted well. The patient could be withdrawn from
766 his analgesics. The Broviac catheter was removed and the patient was discharged 7 ½
767 months after admission. At this time, he still had minor defects on the right thigh and the
768 buttocks (Fig. 1 and Extended Data Fig. 2). The iscorEB clinical score was 12. The
769 transplanted skin was clinically stable and not forming blisters. The child returned back to
770 regular elementary school on March 2016.
771
772 **Cell lines.**
773 **3T3J2 cell line.** Mouse 3T3-J2 cells were a gift from Prof. Howard Green, Harvard
774 Medical School (Boston, MA, USA). A clinical grade 3T3-J2 cell bank was established
775 under GMP standards by a qualified contractor (EUFETS, GmbH, Idar-Oberstein,
776 Germany), according to the ICH guidelines. GMP-certified 3T3-J2 cells have been
777 authorized for clinical use by national and European regulatory authorities and cultured in
778 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% irradiated calf
779 serum, glutamine (4 mM) and penicillin-streptomycin (50 IU/ml).
780 **MFG-LAMB3-Packaging cell line.** A retroviral vector expressing the full-length 3.6-kb
781 *LAMB3* cDNA under the control of the MLV LTR was constructed by cloning a 3.6-kb of
782 LAMB3 cDNA (Gene Bank Accession #Q13751) into MFG-backbone [13]. A 5' fragment of
783 LAMB3 cDNA (563bp) from the ATG to StuI site was obtained by PCR using as template
784 the LB3SN plasmid [33]. The PCR product was cloned into NcoI and BamHI sites of MFG-
785 vector. The second fragment of LAMB3 cDNA (3050bp) was obtained from LB3SN by

786 enzyme digestion from StuI to XmnI and cloned into MGF-vector into StuI site. The entire
787 cDNA of LAMB3 was fully sequenced. The Am12-MGFLAMB3 producer cell lines were
788 generated by transinfection in the amphotropic Gp+*env*Am12 packaging cell line [34].
789 Briefly, plasmid DNA was introduced into the GP+E86 ecotropic packaging cell line [34] by
790 standard calcium phosphate transfection. Forty-eighth ours after transfection, supernatant
791 was harvested and used to infect the amphotropic packaging cell line GP+envAml2 ATCC
792 n° CRL 9641 [13] for 16h in the presence of 8 ug/ml Polybrene. Infected Am12 cells were
793 clonally selected in HXM medium supplemented with 10% FCS, and containing 0.8mg/ml
794 G418 and 0.2mg/ml hygromycin B (Sigma). Single colonies were screened for human
795 LAMB3 production by immunofluorescence using an antibody specific for LAMB3 6F12
796 monoclonal antibody (from Dr. Patricia Rousselle, CNRS, Lyon) and for viral titer. The
797 resulting producer cell lines showed a viral titer of $2X10^6$ colony-forming units (cfu). A
798 master cell bank of a high-titer packaging clone (Am12-*LAMB3* 2/8) was made under GMP
799 standards by a qualified contractor (Molmed S.p.A, Milan, Italy) according to the ICH
800 guidelines and cultured in DMEM supplemented with 10% irradiated fetal bovine serum,
801 glutamine (2 mM), and penicillin-streptomycin (50 IU/ml). All certifications, quality and
802 safety tests (including detection on viruses and other micro-organisms both *in vitro* and *in*
803 *vivo*) were performed under GMP standards for both cell lines.
804
805 **Generation of genetically corrected epidermal sheets and graft preparation.** Primary
806 cultures were initiated from skin biopsy taken from a non-blistering area of inguinal region.
807 Transgenic cultured epidermal grafts were prepared under GMP standards by Holostem
808 Terapie Avanzate S.r.l. at the Centre for Regenerative Medicine "*Stefano Ferrari*",
809 University of Modena and Reggio Emilia, Modena, Italy. Briefly, a 4-cm^2 skin biopsy was
810 minced and trypsinized (0.05% trypsin and 0.01% EDTA) at 37°C for 3h. Cells were
811 collected every 30 min, plated ($2.7x10^4$ cells/cm^2) on lethally irradiated 3T3-J2 cells
812 ($2.66x10^4$ cells/cm^2) and cultured in 5% CO_2 and humidified atmosphere in keratinocyte
813 growth medium (KGM): DMEM and Ham's F12 media (2:1 mixture) containing irradiated
814 fetal bovine serum (10%), insulin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.4
815 μg/ml), cholera toxin (0.1 nM), triiodothyronine (2 nM), glutamine (4 mM), epidermal growth
816 factor (10 ng/ml), and penicillin-streptomycin (50 IU/ml). Sub-confluent primary cultures
817 were trypsinized (0.05% trypsin and 0.01% EDTA) at 37°C for 15-20 minutes and seeded
818 ($1.33x10^4$ cells/cm^2) onto a feeder-layer ($8x10^4$ cells/cm^2) composed of lethally irradiated
819 3T3-J2 cells and producer GP+*env*Am12-*LAMB3* cells[12] (a 1:2 mixture) in KGM. After 3

820 days of cultivation, cells were collected and cultured in KGM onto a regular 3T3-J2 feeder-
821 layer. Sub-confluent transduced cultures were pooled, re-suspended in KGM
822 supplemented with 10% glycerol, aliquoted, and frozen in liquid nitrogen (36 vials, $5x10^6$
823 cells/vial). At each step, efficiency of colony formation (CFE) by keratinocytes was
824 determined by plating 1000 cells, fixing colonies with 3.7% formaldehyde 12 days later and
825 staining them with 1% Rhodamine B.
826 For the preparation of plastic-cultured grafts, transduced keratinocytes were thawed and
827 plated ($1x10^4$ cells/cm^2) on 100 mm culture dishes containing lethally irradiated 3T3-J2
828 cells and grown to confluence in KGM with no penicillin-streptomycin. Grafts were then
829 detached with Dispase II, 2.5 mg/ml (Roche Diagnostics S.p.a.) and mounted basal side
830 up on sterile non-adhering gauze (Adaptic, Systagenix Wound Management, Gargrave,
831 UK). For fibrin-cultured grafts, fibrin gels were prepared in 144 cm^2 plates (Greiner,
832 Stuttgart, Germany) as described[10,12,35]. Fibrin gels consisted of fibrinogen (23.1 mg/ml)
833 and thrombin (3.1IU/ml) in NaCl (1%), $CaCl_2$ (1mM) and Aprotinin (1786 KIU/ml).
834 Transduced keratinocytes were thawed and plated ($1x10^4$ cells/cm^2) on lethally irradiated
835 3T3-J2 cells onto the fibrin gels and grown as above. Grafts were washed twice in DMEM
836 containing 4 mM glutamine, and placed in sterile, biocompatible, non-gas-permeable
837 polyethylene boxes containing DMEM and 4 mM glutamine. Boxes were closed, thermo-
838 sealed and packaged into a sealed, sterile transparent plastic bag for transportation to the
839 hospital.
840

841 **Immunofluorescence (IF), In situ hybridization (ISH), transmission electron**
842 **microscopy (TEM), Hematoxylin/Eosin staining and indirect immunofluorescence**
843 **(IIF).**

844 The following antibodies were used for IF: mouse 6F12 monoclonal antibody to laminin
845 332-β , laminin 332-α3 BM165 mAb (both from Dr. Patricia Rousselle, CNRS, Lyon),
846 laminin 332-γ2 D4B5 mAb (Chemicon), α6 integrin 450-30A mAb and β4 integrin 450-9D
847 mAb (Thermo Fisher Scientific).
848 For immunofluorescence, normal skin biopsies were obtained as anonymized surgical
849 waste, typically from abdominoplasties or mammoplasty reduction and used as normal
850 control. Ethical approval for obtaining the tissue, patient information sheets, and consent
851 forms have been obtained and approved by our institutions (Comitato Etico Provinciale,
852 Prot. N° 2894/C.E.). The patient's skin biopsies were taken randomly, upon agreement
853 patient information sheets and consent forms, at 4, 8 and 21 months. Skin biopsies were

854 washed in PBS, embedded in Killik-OCT (Bio-Optica) and frozen. Immunofluorescence
855 was performed on 7μm skin sections (fixed in PFA 3%, permeabilized with PBS/triton 0.2%
856 for 15 min at r.t. and blocked 1h at r.t with BSA 2% in PBS/triton 0.2%) using the previous
857 described antibodies in BSA 2% in PBS/triton 0.2% and added to skin sections for 30 min
858 at 37°C. Sections were washed 3 times in PBS/triton 0.1% and incubated with Alexa Fluor
859 488 goat anti-mouse (Life Technologies), diluted 1:2,000 in BSA 2%, PBS/triton 0.2% for
860 30 min at 37°C. Cell nuclei were stained with DAPI. Glasses were then mounted with Dako
861 Mounting medium and fluorescent signals were monitored under a Zeiss confocal
862 microscope LSM510meta with a Zeiss EC Plan-Neofluar 40x/1.3 oil immersion objective.
863 To assess the percentage of transduced colonies, 10,000 cells from the sub-confluent
864 transduced PGc pool were plated on a chamber slide and cultivated for 5 days as above.
865 Chamber slides were fixed in methanol 100% for 10 min at -20°C and
866 immunofluorescence analysis was performed as above. Laminin 332-β positive colonies
867 were counted under a Zeiss Microscope AXIO ImagerA1 with EC-Plan Neofluar 20x/0.5
868 objective.
869 *In situ* hybridization (ISH) was performed on 10μm skin sections. DIG-RNA probe
870 synthesis was performed according to the manufacturer's instructions (Roche, DIG
871 Labelling MIX). Primer pairs with Sp6/T7 promoter sequences (MWG Biotech) were used
872 to obtain DNA templates for in vitro transcription. The following vector-specific primers
873 were used: 5'-Sp6-AGTAACGCCATTTTGCAAGG-3' (Tm 60°C) and 5'-T7-
874 AACAGAAGCGAGAAGCGAAC-3' (Tm 58°C) [11,12]. OCT sections were fixed in PFA 4%
875 and permeabilized with proteinase K 5μg/ml and post-fixed in PFA 4%. Sections were then
876 incubated in hybridization solution (50% formamide, 4x SSC, Yeast RNA 500 μg/ml, 1x
877 Denhard's solution, 2 mM EDTA, 10% dextran sulfate in DEPC treated water) at 37°C for 1
878 h. DIG-probes were diluted in pre-heated hybridization solution at 80°C for 2 min and
879 added to the slice for 20 h at 37°C. Sections were washed, blocked in Antibody buffer (1%
880 blocking reagent from Roche in PBS tween 0.1%) containing 10% sheep serum for 1 h at
881 RT. Anti-DIG antibody 1:200 was diluted in the same blocking solution and added to the
882 slide for 4 h at room temperature. Signals were developed with BM-Purple solution ON at
883 RT until signal reached the desired intensity. Slices were then mounted in 70% glycerol
884 and visualized with Zeiss Cell Observer microscope with EC-Plan Neofluar 20x/0.5
885 objective.
886 For transmission electron microscopy, skin biopsies were fixed in 2.5% glutaraldehyde in
887 Tyrode's saline pH 7.2 (24 hr at 4C°), post fixed in 1% osmium tetroxide (Electron

888 Microscopy Sciences) for 2 hr at room temperature, dehydrated in ethanol and propylene
889 oxide, and embedded in Spurr resin (Polysciences). Ultrathin 70nm-thick sections were
890 collected on copper grids, stained with uranyl acetate and lead citrate, and observed with a
891 Jeol 1200 EXII (Jeol Ltd, Akishima, Japan) electron microscope.
892 For H&E staining, sections (7μm) were stained with H&E (Harris hematoxylin for 2 min,
893 running tap water for 1 min, eosin Y for 2 min, 70% ethanol for 1 min, 95% ethanol for 1
894 min, 100% ethanol for 1 min, two rinses in 100% xylene for 1 min each) and observed with
895 Zeiss Microscope AXIO ImagerA1 with EC-Plan Neofluar 20x/0.5 objective.
896 For indirect IF, normal human skin and monkey esophagus sections (Menarini – Trinity
897 Biotech, Buffalo, USA), were incubated with the patient's plasma (diluted 1:10) or with
898 healthy donor plasma as a negative control. Bound human IgG on monkey esophagus
899 sections was detected using α-human IgG monkey-adsorbed, FITC labelled antibody
900 (Inova Diagnostics, San Diego, USA) and on a normal human, split-skin using α-human
901 IgG, FITC labelled antibody (Menarini - Trinity Biotech, Buffalo, USA). Positive control
902 sections were stained with a polyclonal rabbit anti-human laminin-332 antibody (Seralab,
903 West Sussex, United Kingdom) (1:100), and goat α-mouse IgG FITC (Millipore/Merck,
904 Vienna, Austria) secondary antibody. Mounting medium and fluorescent signals were
905 monitored under an Axio Observer D1 Objektiv LD Plan-NEOFLUAR 20X/0,4 Ph2 Korr ∞
906 /0-1.5.
907
908 **Clonal Analysis and DNA Analysis**. Clonal analysis was performed as described[34] and
909 shown in Extended Data Fig. 5. Sub-confluent epidermal cultures were trypsinized, serially
910 diluted and plated in 96 wells plates (0.5 cells/well). After 7 d of cultivation, single clones
911 were identified under an inverted microscope and trypsinized. A quarter of the clone was
912 cultured for 12 days onto a 100 mm (indicator) dish, which was then fixed and stained with
913 Rhodamine B for the classification of clonal type[3]. The remaining part of the clone (3/4)
914 was cultivated on 24-multiwell plates for genomic DNA extraction and further analysis
915 (Extended Data Fig. 5).
916
917 **Library preparation and sequencing.** Illumina barcoded libraries were obtained from 3
918 independent pre-graft cultures (PGc, generated by 3 vials, each containing ~220,000
919 clonogenic keratinocytes) and 3 post-graft cultures (4Mc, $8Mc_1$, and $8Mc_2$). For each
920 sample, 2 tubes with 500 ng of genomic DNA were sheared in 100 μl of water applying 3
921 sonication cycles of 15 sec/each in a Bioruptor (Diagenode) to obtain fragments of 300-

500 bp. Fragmented DNA was recovered through purification with 0.8 volumes of Agencourt AMPure XP beads, two washing steps with 80% ethanol, and elution in Tris-HCl 10 mM. Repair of DNA ends and A-tailing of blunt ends were both performed using Agilent SureSelect^{XT} reagents (Agilent Technologies), according to manual specifications, followed by purification with 1.2 volumes of AMPure XP beads. A custom universal adapter was generated by annealing <Phos-TAGTCCCTTAAGCGGAG - C3> oligo and <GTAATACGACTCACTATAGGGCNNNNNNCTCCGCTTAAGGGACTAT> oligo on a thermocycler from 95°C to 21°C, with decrease of 1°C/min in a 10 mM Tris-HCl, 50 mM NaCl buffer. Ligation of universal adapter to A-tailed DNA was carried out in a reaction volume of 30µl with 400 U of T4 DNA ligase (New England Biolabs) with respective T4 DNA ligase buffer 1X and 35 pmol of dsDNA universal adapter and incubated at 23°C for 1 h, at 20°C for 1 h, and finally heat inactivated at 65° C for 20 min. Each ligation product was purified with 1.2 volumes of AMPure XP beads as described above. Eluate of each reaction was split in 3 different tubes to perform independent PCR reaction in order to mitigate reaction-specific complexity reduction. Each tube was amplified by PCR with a combination of I7-index primers (701/702/703), to multiplex samples on the same Illumina sequencing lane, and of two I5 LTR-primers (501/502) to barcode specific enrichments of MLV-LTR sequences (Supplementary Table 1). PCR reaction was carried out in a final volume of 25 µL, with 20 pmoles of each primer and Phusion High-Fidelity master mix 1X (New England Biolabs). PCR products were purified with 0.8 AMPure XP beads and all amplification products from the same sample (2 fragmentations, 3 PCR reactions) were pooled and quantified on Bioanalyzer 2100 high sensitivity chip. Paired-end 125 bp sequencing was performed on Illumina HiSeq2500 (V4 chemistry). Illumina barcodes on the whole Illumina lanes were combined to maintain a minimum hamming-distance of at least 3 nucleotides. Extraction and de-multiplexing of reads was obtained using CASAVA software (v. 1.8.2) applying a maximum barcode mismatch of 1 nucleotide and considering the dual indexing of I7-I5 sequences. Reads were processed using the bioinformatics pipeline described in details in the Methods. Briefly, reads were first inspected with cutadapt[36] to verify specific enrichments, then trimmed using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and bbduk2 (http://jgi.doe.gov/data-and-tools/bbtools/) to remove adaptors and primers, and mapped to the human genome reference sequence GRCh37/hg19 using BWA MEM[37] with default parameters and the -M flag. Finally, the start coordinate of the alignment was used as the putative integration site.

956 **Genomic and functional annotation of integration events.** Annotation of integration
957 sites to gene features was performed using the *ChIPseeker* R package[36]. Insertion sites
958 were mapped to promoters (defined as 5 kb regions upstream of the transcription start
959 site), exons, and introns of RefSeq genes, and intergenic regions. Functional enrichment
960 in GO Biological Processes of genes harboring an integration site was performed using the
961 *clusterProfiler* R package[36], setting a q-value threshold of 0.05 for statistical significance.
962 Annotation of integration sites to epigenetically defined transcriptional regulatory elements
963 was performed with the BEDTools suite [38] using publicly available ChIP-seq data of
964 histone modifications (H3K4me3, H3K4me1, and H3K27ac) in human keratinocyte
965 progenitors (GSE64328)[36].
966
967 **Linear amplification-mediated (LAM) PCR, NGS on holoclones, PCR on**
968 **mero/paraclones and integration site analysis.** 100 ng of DNA of transduced
969 keratinocytes was used as template for LAM-PCR. LAM-PCR product was initiated with a
970 50-cycle linear PCR and digested with 2 enzymes simultaneously without splitting the DNA
971 amount using 1µl MseI (5U/µl) and 1µl PstI (5U/µl) (Thermo Fisher, Waltham, US) and
972 ligation of a MseI restriction site–complementary linker cassette. LAM-PCR was digested
973 with 2 enzymes simultaneously without splitting the DNA amount. The second enzyme PstI
974 was introduced to eliminate the undesired 5´LTR-LAMB3 sequences. The first exponential
975 biotinylated PCR product was captured via magnetic beads and reamplified by a nested
976 second PCR. LAM-PCR primers for MLV-*LAMB3* used are in table 2. For the initial LAM-
977 PCR, the 5′ -biotinylated oligonucleotide complementary to the 3´-LTR sequence (5´-
978 GGTACCCGTGTATCCAATAA-3´) was used for the linear amplification step. The 2
979 sequential exponential amplification steps were performed with nested oligonucleotides
980 complementary to the 3´- LTR sequence (5´- GACTTGTGGTCTCGCTGTTCCTTGG-3´);
981 (5´-GGTCTCCTCTGAGTGATTGACTACC-3´), each coupled with the oligonucleotides
982 complementary to the linker cassette (Supplementary Table 2). LAM-PCR amplicons were
983 either separated on 2% standard agarose gels (Biozym, Hessisch Oldendorf, Germany)
984 and the excised bands cloned into the StrataClone PCR Cloning Kit (Agilent Technologies,
985 Santa Clara), PCR-purified using High Pure PCR Product Purification Kit (Roche, Basel,
986 Switzerland), shotgun cloned, and sequenced by Sanger, or used as unpurified PCR
987 product as template for NGS library preparation. The fragments were end-repaired,
988 adaptor-ligated, nick-repaired and purified by using the Ion Plus Fragment Library Kit (Life
989 Technologies, Carlsbad, US). The template preparation and the sequencing run on the

machine were also performed according to the protocols of Life Technologies. A mean vertical coverage was planned to reach at least 2000 reads.
Screening of the integration sites of the meroclones and paraclones was done by PCR using a combination of the FW primer MLV 3´LTR control F (5'-GGACCTGAAATGACCCTGTG-3') of the LTR and a specific reverse primer (Supplementary Table 3) in the proximity of the integration site. Genomic DNA from the holoclones was used as positive controls.

Provirus copy number (PCN) TaqMan PCR analysis was performed with TaqMan Universal PCR Master Mix and vector-specific *LAMB3* and *GAPDH* probes (*LAMB3*: Hs00165078_m1; *GAPDH*: Hs03929097_g1, Applied Biosystems). The amplicon for *LAMB3* was located between adjacent exons to recognize only provirus *LAMB3*. Reactions were performed with ABI Prism 7900 Sequence Detection System (Applied Biosystems), using 10 ng of genomic DNA. The relative quantity that relates the PCR signal of the target provirus was normalized to the level of *GAPDH* (internal control gene) in the same genomic DNA by using the $2^{-\Delta\Delta CT}$ quantification.

Bioinformatics analysis of sequencing data.
To process the sequencing reads we assembled a custom bioinformatics pipeline composed of standard tools for NGS data analysis. In particular, we first used cutadapt (v1.14; https://cutadapt.readthedocs.io/en/stable/)[36] to verify the presence, in read pairs, of specific sequences indicative of a successful enrichment. Specifically, in the read harboring the I5 LTR-primer sequence (read 1), we searched for the primer sequence and, at its 3'-end, for the remainder LTR sequence. Instead, in the read harboring the I7 indexing primer (read 2), we searched for the presence of the common adapter sequence preceding the 6 indexing bases. Pairs containing both sequences were retained for analysis after trimming the I5 primer and the remainder LTR sequence in read 1 and the common adapter sequence in read 2. Then, we used FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) to remove from read 2 the first 6 indexing bases, utilized as de-duplicator component during de-multiplexing. Since half of the amplification products are expected to be non-informative in the detection of the insertion site, given the identity of the two LTRs of the MLV genome, we applied bbduk2 (http://jgi.doe.gov/data-and-tools/bbtools/) to identify and remove read pairs representing inward-facing LTR primer enrichment events. In bbduk2 we set the kmer length to 27 (k=27) and the edit

1024 distance and the maxbadkmers parameters both to 1. Reads were aligned on the human
1025 genome reference sequence GRCh37/hg19 using BWA MEM [37] with default parameters
1026 and the -M flag (to include multiple-mapping signature in the BAM file). Read pairs sharing
1027 the same mapping coordinates and the same de-duplicator component were labeled as
1028 PCR duplicates and removed. Aligned read pairs were further filtered to retain only those
1029 mapping at a distance comprised between 150 and 600 bp (corresponding to the expected
1030 library insert size), allowing a maximum of 1 bp soft-clip (unaligned) on all ends, with the
1031 exception of the 5' end of read 2 where we allowed 20 bp soft clip since it contains the 18
1032 bp untrimmed common adapter sequence. Finally, we retained read 1 sequences with a
1033 minimum mapping quality of 40 and extracted and counted the alignment coordinates of
1034 their first base, representing the putative insertion site. Insertion sites within 10 bp from
1035 one another were treated as a single insertion, their counts summed using BEDTools
1036 (v2.15; http://bedtools.readthedocs.io/en/latest/content/bedtools-suite.html) [38], and the
1037 summed count assigned to left coordinate. When intersecting insertion sites across
1038 samples, we considered overlapping those insertion events closer than 30bp.

1039

1040 **Calculation of the expected number of integrations.**

1041 The expected number of integrations (i.e., the expected population size) in PGc, 4Mc,
1042 $8Mc_1$, and $8Mc_2$ samples was calculated in R applying a capture-recapture model based
1043 on the Chapman's estimate and its confidence intervals [15,39]:

1044

$$\hat{N} = \frac{(n_1+1)(n_2+1)}{n_{11}+1} - 1$$

$$\hat{N} \pm Z_{1-\alpha/2}\sqrt{\frac{(n_1+1)(n_2+1)n_{21}n_{12}}{(n_{11}+1)^2(n_{11}+2)}}$$

1045

1046 where $\hat{N}$ is the estimated number of integrations, n_1 is the number of integrations found in
1047 the 3pIN library, n_2 those found in the 3pOUT library, n_{11} the number of overlapping
1048 integrations, n_{12} and n_{21} the insertion respectively exclusive of 3pIN and 3pOUT,
1049 respectively, and $Z_{1-\alpha/2} = 2.56$ for α=0.01.

1050

1051 **Genomic and functional annotation of insertion events.**

1052 To annotate the integration sites to gene features, we used the *ChIPseeker* R package
1053 (v1.10.3, https://bioconductor.org/packages/release/bioc/html/ChIPseeker.html) [40]. The
1054 integration sites were mapped to promoters, defined as 5 kb regions upstream of

transcription start sites (TSS), exons, and introns of RefSeq genes, and to intergenic regions.

We performed functional annotation of genes harboring an integration sites using the *clusterProfiler* R package (v3.2.14; https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html) [41], setting a q-value threshold of 0.05 to define enriched Gene Ontology (GO) Biological Processes.

To annotate the integration sites to epigenetically defined transcriptional regulatory elements (promoters and enhancers), we used the BEDTools suite (v2.15; http://bedtools.readthedocs.io/en/latest/content/bedtools-suite.html) [38]. We define promoters and enhancers using publicly available ChIP-seq data of histone modifications (H3K4me3, H3K4me1 and H3K27ac) produced in human keratinocyte progenitors [42]. Briefly, bed files containing the coordinates of genomic regions enriched for each histone modification (peaks) were downloaded from the Gene Expression Omnibus database (GSM1568245 for H3K4me3, GSM1568244 for H3K4me1 and GSM1568247 for H3K27ac). H3K4me3 peaks close to the TSS (<5 kb) of RefSeq genes were defined as promoters, while H3K4me1 peaks far from TSS (>5 kb) were defined as enhancers. Promoters and enhancers were classified as “active” if they overlap with H3K27ac peaks, otherwise are classified as “weak”. Finally, integration sites were mapped to active and weak promoters and enhancers.

Differences in the annotation of integration sites to gene features and regulatory elements were tested using the *chisq.test* function (Pearson's Chi-squared test) of the *stat* R package.

Bioinformatics analysis of NGS data from holoclones.

Analysis of the data was implemented with single read sequences of the BAM file. Output results with ≥ 5% of query cover, ≥ 95% identity, and a size of ≥ 48bp were taken into account for confirming as integration site with control PCR. Sequences were aligned to the human genome (*Genome Reference Consortium GRCh37*) using the NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Identification of the nearest gene was performed with dedicated PERL scripts. Visualization of the RTCGD CIS integrations as a feature on the UCSC BLAT output was achieved by connecting to UCSC through the RTCGD web interface (http://rtcgd.abcc.ncifcrf.gov); map position of each of the retroviral integrations was automatically loaded as custom tracks on the UCSC BLAT search engine.

Statistical analyses and data visualization. Statistical analyses were implemented in R (v3.3.1, http://www.r-project.org/). Figure 3d was generated using the *ggplot2* R package (v2.2.1, https://cran.r-project.org/web/packages/ggplot2/index.html).

Data availability. All high-throughput sequencing data of the integration profiles have been deposited in the Sequence Read Archive (SRA) under accession number SRP110373. All data used to generate main and supplementary figures are provided as source data files.

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