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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.10.036.

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Closure of a Large Chronic Wound through Transplantation of Gene-Corrected Epidermal Stem Cells

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TO THE EDITOR

Generalized junctional epidermolysis bullosa (JEB) is caused by mutations in *LAMA3, LAMB3,* or *LAMC2,* which together encode laminin-332, a hetero-trimeric protein consisting of α3, β3, and γ2 chains (Fine et al., 2014). In nonlethal generalized intermediate JEB, laminin-332 is highly reduced, and hemidesmosomes are rudimentary or completely absent, leading to blister formation within the lamina lucida of the basement membrane upon minor trauma. The resulting chronic skin wounds invariably develop recurrent infections and scarring, which greatly impair patients' quality of life (Fine et al., 2014; Laimer et al., 2010; Nakano et al., 2002).

There is no cure for JEB; treatments are symptomatic and aimed at relieving the devastating clinical manifestations (Carulli et al., 2013). The only published evidence for the possibility of a permanent local treatment of JEB was provided by a phase I/II trial showing that autologous epidermal cultures containing genetically modified epidermal stem cells were able to restore a normal epidermis on a JEB patient (De Rosa et al., 2014; Mavilio et al., 2006). However, the transgenic epidermis was applied in areas still covered by a diseased but apparently functional epidermis, which was surgically removed before grafting (Mavilio et al., 2006). Although it is clear that the ideal clinical application of transgenic

epidermis would aim at preventing the development of devastating chronic lesions, many patients suffer from therapy-resistant chronic ulcerations that are highly predisposed to cancer development and need timely closure (Goldberg et al., 1988; Hoste et al., 2015). We report on a patient in whom gene-corrected epidermal sheets were transplanted onto a large nonhealing epidermal ulceration following a good manufacturing practice protocol. This single-case study was authorized by the Austrian Ministry of Health, and all experiments were approved by the University Hospital of the Paracelsus Medical University, Salzburg. Written informed consent was given by the patient, who also consented on the publication of photographs and medical information.

A 49-year-old woman with generalized intermediate, laminin-332-β3−dependent JEB presented with a large (approximately 80-cm^2) wound on her lower right leg (Figure 1a)
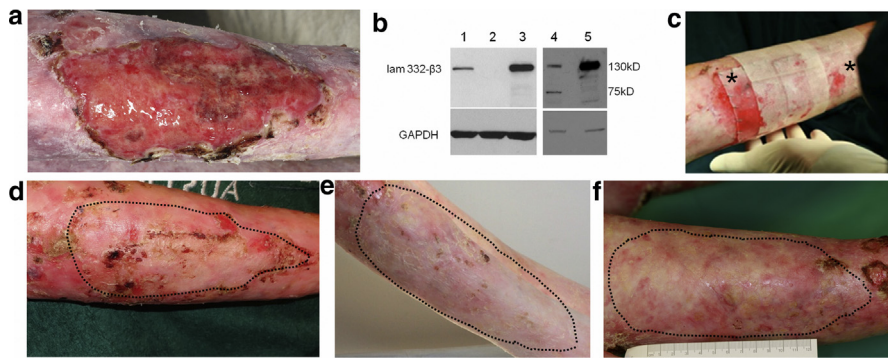
Figure 1. Regeneration of a transgenic functional epidermis on the skin wound of the JEB patient. (**a**) The long-standing ulceration on the lower right leg of the patient 2 days before transplantation. (**b**) Western blot analysis of cell lysates (20 µg protein, 30 seconds exposure time) from (lane 1) normal control and patient keratinocyte cultures (lane 2) before and (lane 3) after gene correction, probed with a monoclonal antibody against laminin 332-β3. (lane 4) Western blot analysis of a higher amount of loaded protein (65 µg, 5 seconds exposure time) of uncorrected patient keratinocytes, and (lane 5) normal keratinocyte cultures using the same laminin-332-β3 antibody. The 75-kD band in lane 4 is consistent with the truncated laminin-332-β3 generated by the c.1903C>T; p.R635X mutation. (**c**) Transplantation of cultured transgenic epidermal sheets (asterisks) on the prepared wound bed. Grafts are overlaid with petrolatum gauze. (**d**) Initial epidermal regeneration at 14 days. (**e**) Complete epidermal regeneration at 3.5 months. (**f**) Stable epidermal regeneration at 16 months. Note crusting and erosions outside of the grafted area. JEB, junctional epidermolysis bullosa.

(Buchroithner et al., 2004). Whereas recurrent blisters covering her body have been treated with conventional therapies with varying success, the large lesion on her right leg persisted for over 10 years, and recurrent infections led at one point to post-streptococcal glomerulonephritis. In July 2014 her ulceration was treated with a combined ex vivo gene- and autologous cell-therapy approach. Primary patient keratinocytes, obtained from a biopsy of the palm, were transduced by the same retroviral vector (RV) expressing the full-length *LAMB3* cDNA under the control of the Moloney leukemia virus (MLV) long-terminal repeat, as used in the previous study (Mavilio et al., 2006), at an efficiency of 99.4% and an average of two proviral copies per cell (see Supplementary Figure S1 online). Whereas untransduced patient keratinocytes contained barely detectable amounts of laminin-332-β3 (Figure 1b, lanes 2 and 4), genetically modified cells contained an amount of laminin-332-β3 (Figure 1b, lane 3) even higher than normal keratinocytes (Figure 1b, lane 1). Transgene expression persisted at constant levels throughout the lifespan of the culture (approximately 100 cell doublings, data not shown).

Gene-corrected clonogenic cells ($\sim 4 \times 10^5$) were expanded and used to grow two cohesive epidermal sheets of approximately 80 cm^2 to be transplanted onto the leg ulceration after wound bed preparation (Figure 1c). Complete engraftment of the transgenic epidermis was observed after 14 days (Figure 1d). The skin was apparently normal, with some hyperkeratosis originating from the initial biopsy site, the lateral aspect of the left palm. The graft remained mechanically stable throughout the entire follow-up period (16 months) and without blister formation, even upon shear force by repeated rubbing of the skin (Figure 1e and f).

On histological analysis of skin biopsy samples taken at 1-year follow-up, we observed a normal and fully differentiated epidermis and a normal dermal-epidermal junction (Figure 2b). Immunofluorescence analysis showed that the transgenic epidermis expressed a normal amount of laminin-332 (Figure 2c), comparable to that observed in a normal control (Figure 2d), that is properly located at the dermal-epidermal junction. Transmission electron microscopy showed appropriate morphology of the basement membrane zone in the transplanted area (Figure 2e), unlike the patient's lesional skin before grafting (Figure 2f). In situ hybridization performed using vector-specific *LAMB3* probes showed homogenous expression of *LAMB3* mRNA in all epidermal

layers (Figure 2g), confirming that the regenerated epidermis consists only of transgenic keratinocytes. Because human epidermis is renewed monthly, the patient's transgenic epidermis underwent at least 16 complete renewal cycles during the 16 months of follow-up. Thus, the long-term maintenance of the regenerated epidermis must be due to the engraftment of self-renewing transduced epidermal stem cells. This assumption was confirmed by genome-wide analysis of RV integration sites performed on DNA extracted from a 3-mm^2 punch biopsy sample of the transgenic epidermis at 1-year follow-up. Libraries of vector-genome junctions, generated by ligation-mediated nested PCR and sequenced to saturation, retrieved three independent integrations into genes unambiguously mapped on the human genome (see Supplementary Table S1 online). Quantitative real-time reverse transcriptase—PCR analysis performed on untransduced and transduced primary cultures showed no change in the expression of the three genes, indicating that they were not dysregulated through the proviral integration (data not shown). Given an average integration per cell of 1.5—2.0, as determined by Southern blot analysis and previously shown (Mavilio et al., 2006), these results indicate that the ~150 clonogenic cells present in the small area of the taken biopsy arise from at least two single stem cell clones. These data confirm the notion that the entire regenerated epidermis is sustained only by the engrafted stem cells (De Rosa et al., 2014).

To ensure the safety of our approach, we further demonstrated the complete absence of pathogenic antibodies against the newly synthesized laminin-332-β3 protein by indirect immunofluorescence analysis using the patient's plasma collected 12 months after transplantation (see Supplementary Figure S2 online).

Despite the occurrence of severe adverse events in patients with genetic immunodeficiency treated with MLV-based RV-transduced hematopoietic stem cells (Carulli et al., 2013), these vectors cannot per se be considered oncogenic, because other clinical trials
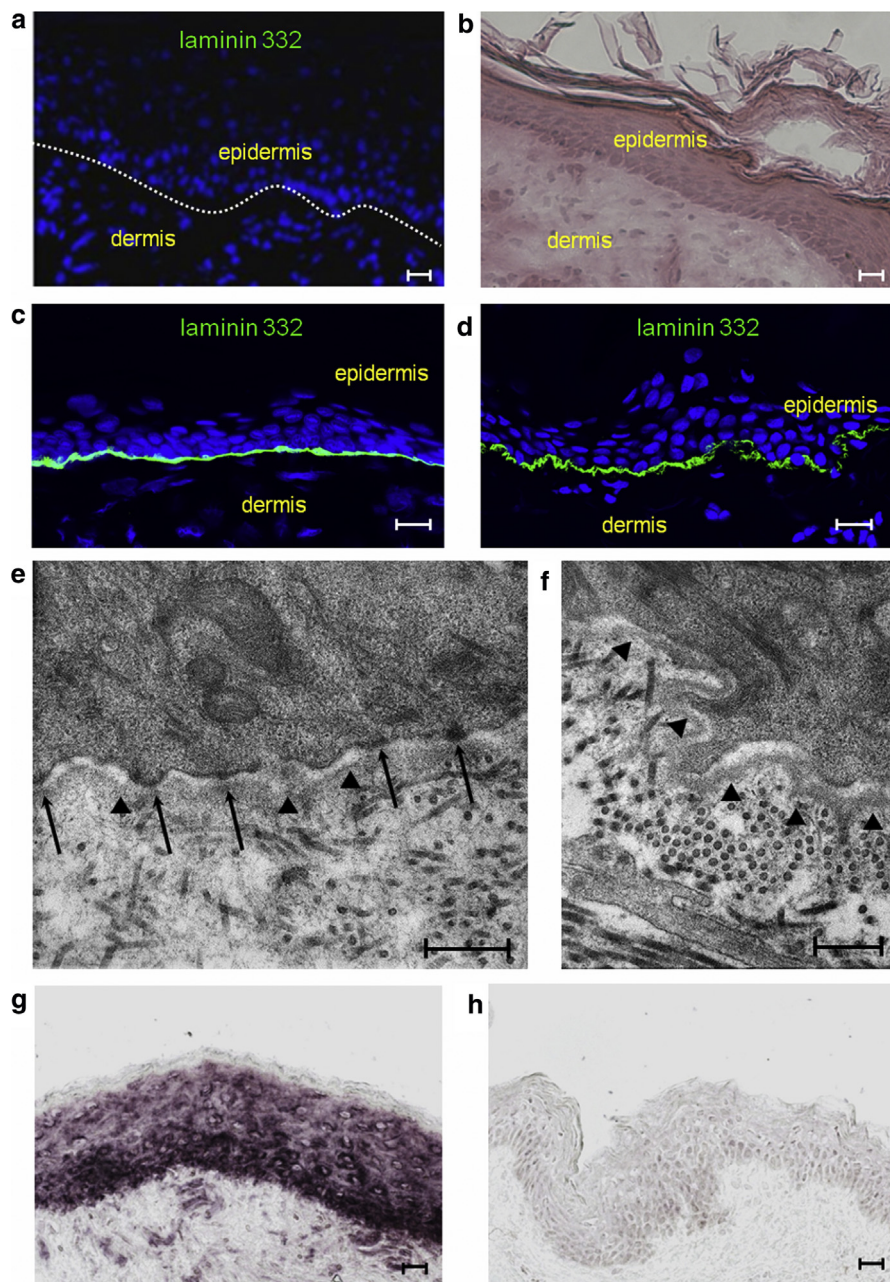
Figure 2. Expression of laminin-332 protein and *LAMB3* mRNA in the regenerated transgenic epidermis and restoration of a normal dermal-epidermal junction. (**a**) Sections (7 μm thick) from the patient's skin before transplantation were immunostained using a polyclonal antibody against laminin-332. (**b**) Hematoxylin and eosin staining of a skin section from a biopsy sample at 1-year follow-up. (**c**) Immunofluorescence staining of laminin-332 in corrected skin after 1 year and (**d**) normal control skin sections. Scale bars = 10 μm. (**e**) Transmission electron microscopy performed on skin biopsy samples from the transplanted area of the patient's leg and (**f**) from the patient's skin before transplantation. Arrowheads indicate basement membrane; arrows show hemidesmosomes. Scale bars = 0.5 μm. In situ hybridization of *LAMB3* mRNA using vector-specific *LAMB3* probes was performed (**g**) in skin sections taken from the transplanted area of the patient's leg at the 1-year follow-up and (**h**) in sections of normal control skin. Cell nuclei were counterstained with DAPI (blue). Dotted line shows dermal-epidermal junction. Scale bars = 20 μm.

using the same vector backbone in the same cell type have not been accompanied by any serious adverse effects (Aiuti et al., 2009). The safety of using an MLV-based RV for skin gene therapy approaches is underscored by the fact that during the 10 years' follow-up of the previously treated JEB patient, the vector did not cause any adverse events (De Rosa et al., 2014). This notion indicates that the risk of vector-induced mutagenesis might require other factors possibly related to cell type, genetic background, disease, and the clinical protocol.

Taken together, our data show that a functional epidermis has been regenerated on a previously infected nonhealing skin ulceration by a discrete number of gene-corrected epidermal stem cells, which might also reduce the risk of cancer development in such treated wounds. This underscores the high therapeutic potential of ex vivo gene therapy in the skin. On the basis of these results, further affected skin areas of the patient in this study can be treated, and the technology can be transferred to treat other JEB and dystrophic epidermolysis bullosa patients.

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SUPPLEMENTARY MATERIAL

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