Regeneration of the entire human epidermis using transgenic stem cells

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Junctional epidermolysis bullosa (JEB) is a severe and often lethal genetic disease caused by mutations in genes encoding the basement membrane component laminin-332. Surviving patients with JEB develop chronic wounds to the skin and mucosa, 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 seven-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 the human epidermis is sustained not by equipotent progenitors, but by a limited number of long-lived stem cells, detected as holoclones, that can extensively self-renew *in vitro* and *in vivo* and 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.

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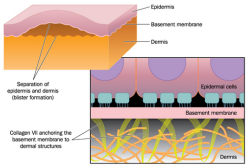
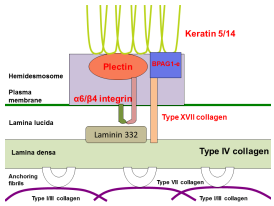
Junctional epidermolysis bullosa (JEB)

- severe and often lethal genetic disease
- structural and mechanical fragility of the integuments, blisters and erosions of the skin and mucosa within the lamina lucida of the basement membrane in response to minor trauma
- massive chronic skin wounds, recurrent infections and scars, predisposition to skin cancer.

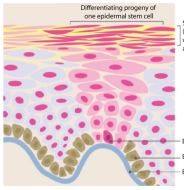


- mutations in three genes—LAMA3, LAMB3 or LAMC2—that jointly encode laminin-332 (a heterotrimeric protein, also known as laminin 5, consisting of α3, β3, and γ2 chains), collagen XVII and α6β4 integrins.

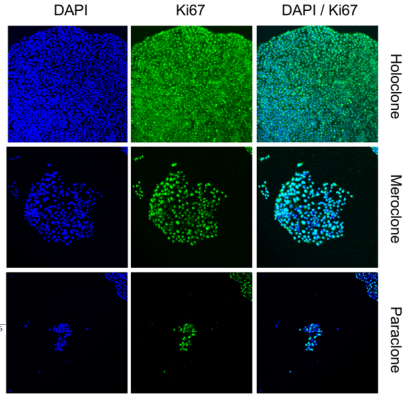


- deleterious mutations that cause an absence of laminin-332 are usually lethal early in life.
- in nonlethal cases, laminin-332 is strongly reduced and hemidesmosomes are rudimentary or absent
- no cure
- 40% of patients die before adolescence

Epidermal stem cells



Monthly renewal and timely repair of the human epidermis is sustained by epidermal stem cells, which generate colonies known as **holoclones**.



Holoclones produce meroclone- and paraclone-forming cells, which behave as transient amplifying progenitors.

Epithelial cultures harbouring holoclone-forming cells can permanently restore massive skin and ocular defects

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Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells

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Owing to the paucity of treated areas (a total of around 0.06 m2), the treatment did not improve the patients' quality of life.

The patient

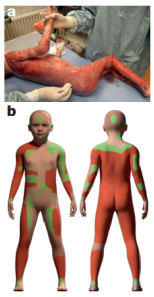
June 2015: a seven-year-old child admitted to the Burn Unit of the Children's Hospital, Ruhr-University, Bochum, Germany.

Homozygous acceptor splice site mutation (C1977-1G> A, IVS 14-1G> A) within intron 14 of LAMB3

Since birth, blisters all over his body, particularly on his limbs, back and flanks. Condition deteriorated severely six weeks before admission, owing to infection with Staphylococcus aureus and Pseudomonas aeruginosa.

Shortly after admission, complete epidermal loss on about 60% of his total body surface area (TBSA). At the time of the first surgery, the patient had complete epidermal loss on approximately 80% TBSA

Informed consent by parents and authorisation by regional regulatory authorities for compassionate use of combined ex vivo cell and gene therapy.



Schematic representation of the clinical picture. *The denuded skin is indicated in red; blistering areas are indicated in green. Flesh-coloured areas indicate currently nonblistering skin. Transgenic grafts were applied on both red and green areas*

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What is compassionate use?

Compassionate use is a way of making available to patients with an unmet medical need a promising medicine which has not yet been authorised (licensed) for their condition.

A medicine can be marketed in the European Union (EU) only after it has been authorised. However, it is sometimes in the interest of patients to have access to medicines before authorisation. Special programmes can be set up to make these medicines available to them under defined conditions. This is known as 'compassionate use'.

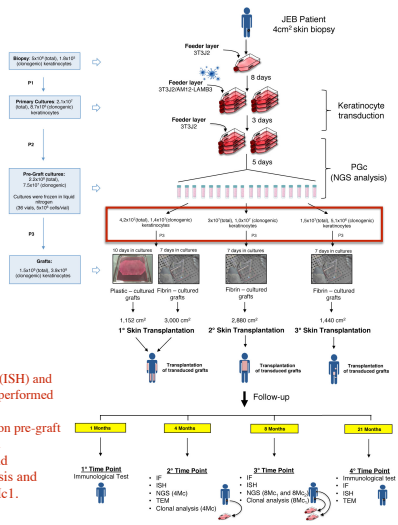
Which medicines can be made available in this way?

Compassionate use programmes can only be put in place for medicines that are expected to help patients with life-threatening, long-lasting or seriously disabling illnesses. These programmes are expected to benefit seriously ill patients who currently cannot be treated satisfactorily with authorised medicines, or who have a disease for which no medicine has yet been authorised. The compassionate use route may be a way for patients who cannot enrol in an ongoing clinical trial to obtain treatment with a potentially life-saving medicine.

At this stage in the development of the medicine, what is known of the medicine's safety may be limited. Generally, toxicology studies will have been completed and analysed, and early studies looking at how the medicine is handled by the body will have been completed. However, there may still be some uncertainties about the best way to give the medicine to patients, such as the exact dose to use, and the dose frequency, and the medicine's safety profile (which side effects it can cause) is not yet fully established.

Regeneration of epidermis by transgenic cultures

A 4-cm² biopsy, taken from a currently non-blistering area of the patient's left inguinal region, was used to establish primary keratinocyte cultures, which were then transduced with a retroviral vector expressing the full-length LAMB3 cDNA under the control of the Moloney leukaemia virus long terminal repeat.



Immunofluorescence (IF), in situ hybridization (ISH) and transmission electron microscopy (TEM) were performed on randomly taken punch biopsies.
Genome-wide analysis (NGS) was performed on pre-graft cultures (PGc) and on primary cultures initiated from biopsies taken from the left leg (4Mc and 8Mc2) and the left arm (8Mc1). Clonal analysis and tracing were performed on PGc, 4Mc and 8Mc1.

Sufficient 0.85-m2 transgenic epidermal grafts, enough to cover all of the patient's denuded body surface, were applied sequentially on a properly prepared dermal wound bed

Transplantation onto the left arm of plastic-cultured epidermal grafts, mounted on a non-adhering gauze (asterisks)

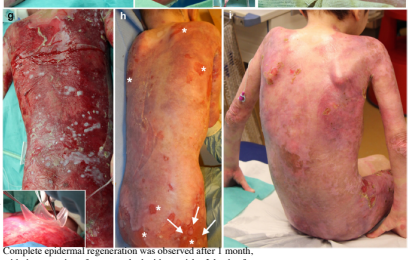
Preparation of a dermal wound bed

The engrafted epidermis (asterisks) is evident upon removal of the gauze (arrows) ten days after grafting.

Regenerated epidermis on the left arm after 1 month.

Transplantation (e) and engraftment (f) of both plastic-cultured (asterisk) and fibrin-cultured (arrow and inset in e) grafts on the left leg.

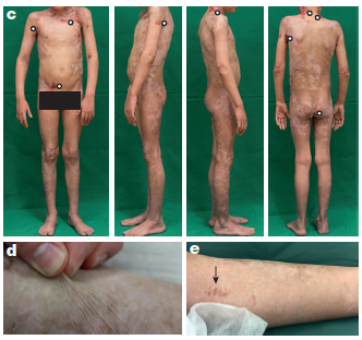
The back of the patient was covered with fibrin-cultured grafts.



Within 4 months, the regenerated epidermis surrounding the open lesions and the epidermal islands detected within those open lesions had spread and covered the denuded areas.

Complete epidermal regeneration was observed after 1 month, with the exception of areas marked with asterisks. Islands of epidermis were observed inside those denuded areas (arrows).

Previously, transgenic epidermal sheets have been cultivated **on plastic**, enzymatically detached from the vessel and mounted on a non-adhering gauze. Keratinocyte cultivation **on a fibrin substrate**—currently used to treat massive skin and ocular burns—eliminates cumbersome procedures for graft preparation and transplantation and avoids epidermal shrinkage, allowing the production of larger grafts from the same number of clonogenic cells as are needed to produce plastic-cultured grafts. Because degradation of fibrin after transplantation, which is critical to allow cell engraftment, had never been assessed in a wound bed of a patient with JEB, at the first surgery we compared plastic- and fibrin-cultured grafts.



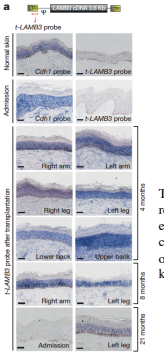
Approximately 80% of the patient's TBSA was restored by the transgenic epidermis.

During the 21-month follow-up (more than 20 epidermal renewing cycles), the regenerated epidermis adhered firmly to the underlying dermis, even after induced mechanical stress, healed normally and did not form blisters, including in areas where follow-up biopsies were taken (arrow).

Ten punch biopsies were taken randomly, 4, 8 and 21 months after grafting. The epidermis had normal morphology without blisters, erosions or epidermal detachment from the underlying dermis

In situ hybridization using a vector- specific t-LAMB3 probe

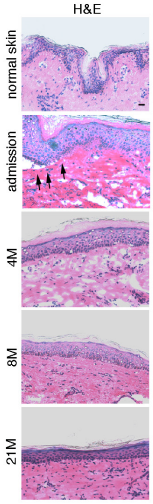
E-cadherin-specific probe (Cdh1) was used as a control.



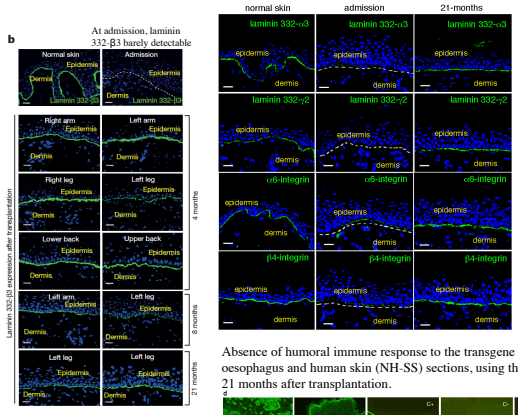
The regenerated epidermis consisted only of transgenic keratinocytes

ruptures at the epidermis–dermis junction

H&E



Transduced keratinocytes restored a proper adhesion machinery

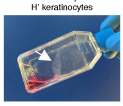


At admission, laminin 332-β3 barely detectable

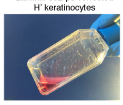
laminin 332-β3properly located at the epidermis–dermis junction

Spontaneous detachment (arrow) of laminin 332-β 3 null keratinocytes from the patient.

Laminin 332-β3 null H' keratinocytes

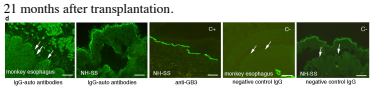


Laminin 332-β3 corrected H' keratinocytes

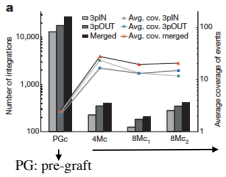


Genetically corrected keratinocytes remained firmly attached to the substrate. As with normal control cells, detachment would require prolonged enzymatic treatment

Absence of humoral immune response to the transgene product on monkey oesophagus and human skin (NH-SS) sections, using the patient's plasma 21 months after transplantation.



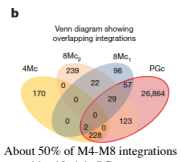
Integration profile of transgenic epidermis



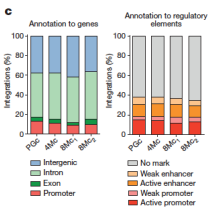
Integrations identified in libraries obtained using two LTR primers: the libraries obtained using the two LTR primers (3pIN and 3pOUT) showed a similar number of reads and comparable insertion counts

biopsies taken 4 and 8 months after grafting: only 400, 206 and 413 integrations (27,000 in PGc)

PG: pre-graft transgenic cultures



About 50% of M4-M8 integrations were identified in PGc



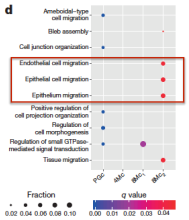
Integrations were mapped to promoters (10%, defined as 5-kb regions upstream of the transcription start site of RefSeq genes), exons (5%), introns (47%), and intergenic regions (38%).

- 27% of integrations associated with active promoters or enhancers

- no significant difference in the distribution of insertions between pre- and post-graft samples

The integration pattern was maintained in vivo and epidermal renewal did not determine any clonal selection.

Genes containing an integration functionally enriched in Gene Ontology categories related to cancer-associated biological processes



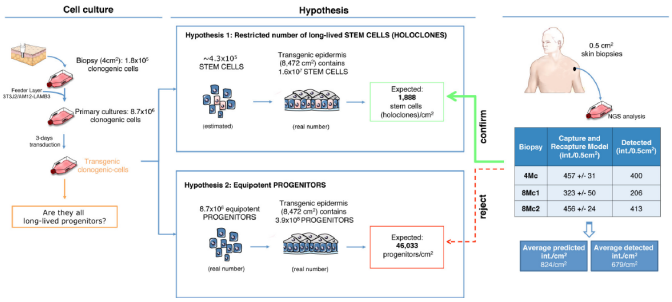
Concerns have been raised about insertional genotoxicity arising from MLV-RV vector use; this has been reported with haematopoietic stem cells in specific disease contexts. A γ RV vector, similar to ours, obtained marketing authorization for ex vivo gene therapy of adenosine deaminase severe combined immunodeficiency and has been approved for phase I/II clinical trials on recessive dystrophic epidermolysis bullosa (RDEB) (https://clinicaltrials.gov/ct2/show/NCT02984085).

The patient's integration profile confirmed the absence of clonal selection both in vitro and in vivo.

Never observed immortalization events related to specific proviral integrations in many serially cultivated MLV-RV-transduced keratinocytes

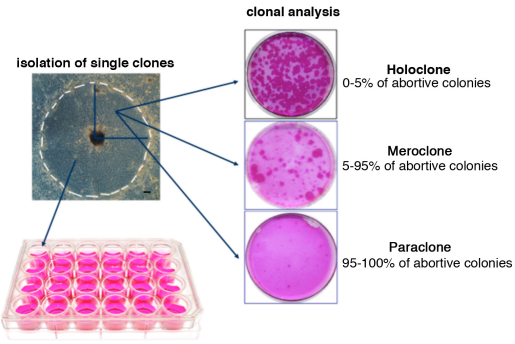
Although the follow-up of this patient was shorter and does not allow us to draw definitive conclusions, the **frequency of detectable insertional mutagenesis events** to date is **less than 1 in 3.9 x10⁸**. In evaluating the risk/benefit ratio, it should also be considered that patients with severe JEB are likely to develop aggressive squamous cell carcinoma as a consequence of the progression of the disease

The transgenic epidermis is sustained by holoclones



If the originally transduced clonogenic cells were equipotent progenitors, we would have recovered thousands of integrations per cm² of regenerated epidermis, and all clonogenic cells contained in 4Mc, 8Mc1 and 8Mc2 cultures would have had independent integrations.
Instead, if the transgenic epidermis were sustained by only a restricted number of stem cells (continuously generating pools of transient amplifying progenitors), we would have recovered only a few hundred integrations, and meroclones and paraclones contained in 4Mc, 8Mc1 and 8Mc2 cultures would have had the same integrations as were found in the corresponding holoclones.
The number of integrations detected in post-graft cultures is consistent with the number of stem cells that have been transplanted, and therefore strongly supports the latter hypothesis, which was verified by proviral analyses at clonal level

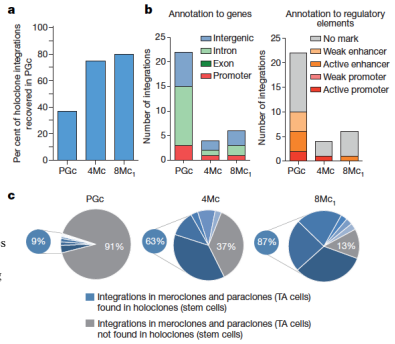
Clonal analysis scheme



Sub-confluent cultures were inoculated (0.5 cells per well) onto 96-multiwell plates containing irradiated 3T3-J2 cells. After 7 days of cultivation, single clones were transferred to two dishes. One dish (one-quarter of the clone) was fixed 12 days later and stained with rhodamine B for the classification of clonal type, determined by the percentage of aborted colonies formed by the progeny of the founding cell.
The second dish (three-quarters of the clone) was used for integration analysis after 7 days of cultivation.

Integration profile of stem and transient amplifying cells

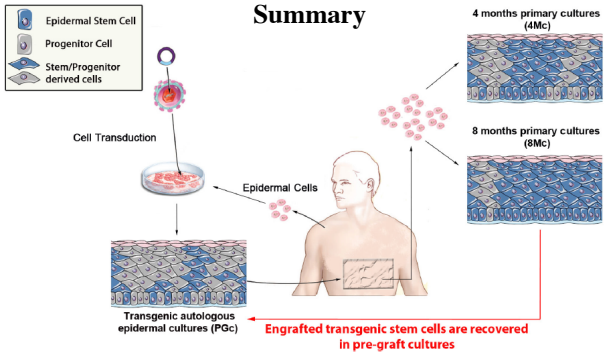
80% of integrations found in 4Mc and 8Mc1 holoclones were retrieved in PGc, supporting the NGS-based survey as well as a representative sampling. The integration pattern observed in holoclones confirms the absence of selection of specific integrations during epidermal renewal in vivo



The majority (91%) of PGc meroclones and paraclones did not contain the same integrations as the corresponding holoclones

This percentage had decreased to 37% by 4 months after grafting and virtually the entire clonogenic population at 8 months contained the same integrations as the corresponding holoclones

Summary



(i) PGc consisted of a mixture of independent transgenic holoclones, meroclones and paraclones
(ii) meroclones and paraclones are transient amplifying progenitors, do not self-renew and are progressively lost during cultivation and in vivo epidermal renewal, and therefore do not contribute to the 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 undergone extensive self-renewal (in vitro and in vivo) to ultimately sustain the regenerated epidermis