

TRANSLATING THE COMBINATION OF GENE THERAPY AND TISSUE ENGINEERING FOR TREATING RECESSIVE DYSTROPHIC EPIDERMOLYSIS BULLOSA

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Abstract

The combination of gene therapy and tissue engineering is one of the most promising strategies for the treatment of recessive dystrophic epidermolysis bullosa (RDEB). RDEB is a rare genetic disease characterised by mutations in the *COL7A1* gene, encoding type VII collagen (COLVII), which forms anchoring fibrils at the dermal-epidermal junction of the skin. This disease causes severe blistering and only palliative treatments are offered. In this study, the base of a strategy combining gene therapy and a tissue-engineered skin substitute (TES), which would be suitable for the permanent closure of skin wounds, was set-up. As a high transduction efficiency into fibroblasts and/or keratinocytes seems to be a prerequisite for a robust and sustained correction of RDEB, different envelope pseudotyped retroviral vectors and the transduction enhancer EF-C were tested. When green fluorescent protein (GFP) was used as a reporter gene to evaluate the retroviral-mediated gene transfer, the fibroblast infection efficiency was 30 % higher with the Amphi pseudotyped vector as compared with the other pseudotypes. At least a 3.1-fold and a 1.3-fold increased transduction were obtained in fibroblasts and keratinocytes, respectively, with EF-C as compared with polybrene. A continuous and intense deposit of haemagglutinin (HA)-COLVII was observed at the dermal-epidermal junction of self-assembled TESs made of cells transduced with a HA-tagged *COL7A1* vector. Furthermore, HA-tagged basal epidermal cells expressing keratin 19 were observed in TESs, suggesting stem cell transduction. This approach could be a valuable therapeutic option to further develop, in order to improve the long-term life quality of RDEB patients.

Keywords: Acantholysis bullosa, epidermolysis bullosa dystrophica, cell- and tissue-based therapy, tissue engineering, cell culture techniques, genetic therapy, collagen type VII.

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Introduction

Epidermolysis bullosa (EB) is a genetic disease comprising a heterogeneous group of rare inherited skin disorders in which minor mechanical stress to the skin and mucous membranes causes the formation of blisters and erosions. There are four major types of EB, characterised by the location of the defective proteins and the level of blisters: EB simplex (EBS; epidermolytic), junctional EB (JEB; lucidolytic), dystrophic EB (DEB; dermolytic) and Kindler syndrome (mixed levels of blistering) (Aumailley *et*

al., 2006; Fine *et al.*, 2014). In the skin of patients with DEB, blister formation occurs beneath the lamina densa within the papillary dermis. Mutations in the *COL7A1* gene are responsible for DEB, which can be autosomal dominant or recessive (RDEB), depending on the type of mutations (Kern *et al.*, 2009). The *COL7A1* gene encodes type VII collagen (COLVII), which is naturally secreted into the extracellular space by dermal fibroblasts and epidermal keratinocytes (Bruckner-Tuderman *et al.*, 1999). These collagen molecules assemble into anchoring fibrils, a major component of the dermal-epidermal junction (DEJ),

responsible for the adhesion between the epidermis and the dermis (Bruckner-Tuderman *et al.*, 1999). DEB is characterised by abnormalities in anchoring fibrils, which are absent, reduced in number or malformed. Patients with RDEB can display other symptoms than blistering and fragility of the skin and mucous membranes; their hands and feet can be deformed and they can suffer from anaemia, malnutrition and growth retardation. The development of squamous cell carcinoma in areas subjected to intense blistering is a common trait in RDEB patients over the age of 30. There is no cure for RDEB and only palliative treatments restricted to individual wounds are currently offered to patients (Aumailley *et al.*, 2006; Rashidghamat and McGrath, 2017; Uitto *et al.*, 2016).

Several experimental strategies for RDEB have been tested in preclinical studies and in patients. These comprise protein therapy, cell therapy (fibroblasts, mesenchymal stromal cells and haematopoietic stem cells), antisense oligonucleotide-mediated exon skipping and gene therapy, including genome engineering (Rashidghamat and McGrath, 2017). The latter approach was also used by Bauer *et al.* (2016) and Mavilio *et al.* (2006) to successfully treat two JEB patients. They cultured keratinocytes, obtained from the patients' own skin, *in vitro* and transduced them with a retroviral vector containing the *LAMB3* gene, which encodes a subunit of laminin 332, another component of the DEJ. After expansion of the corrected cells, they successfully transplanted cultured epidermal sheets onto small areas containing wounds. Very recently, the same team was able to successfully graft 80 % of the body surface of a JEB patient with a transgenic epidermis, a procedure that was life-saving (Hirsch *et al.*, 2017). The transgene was expressed during the 21-month follow-up and the epidermis was fully functional and did not form blisters. This proof of principle suggests that this strategy could be applied to other EB types (De Rosa *et al.*, 2014; Hirsch *et al.*, 2017; Mavilio *et al.*, 2006).

Compared to the *LAMB3* cDNA (3.6 kb) (Mavilio *et al.*, 2006), the large size of the *COL7A1* cDNA (8.9 kb) has limited the generation of high-titer retroviral vectors from stable retrovirus-producer cells (Chen *et al.*, 2002; Goto *et al.*, 2006; Siprashvili *et al.*, 2010). The use of high-titer retroviral packaging cell lines (293Vec cells), constructed in our laboratory, could circumvent the titer issue. They release viral particles pseudotyped with the amphotropic (Ampho), the baboon endogenous virus (Baev), the gibbon ape leukaemia virus (Galv) and the feline endogenous retrovirus (RD114) envelopes (Ghani *et al.*, 2007; Ghani *et al.*, 2009). In the present study, the potential of *GFP* and *COL7A1* vectors, produced from these packaging cell lines, for the transduction of human keratinocytes and fibroblasts was investigated. Also, a 12-amino acid peptide, EF-C, derived from the HIV-1 envelope glycoprotein, was tested. Once assembled in nanofibrils, EF-C is shown to be more potent than the commonly used polycation polybrene for increasing

the transduction of fibroblasts and macrophages (Meier *et al.*, 2014; Yolamanova *et al.*, 2013).

A bilayered skin substitute, with both a dermis and an epidermis, could significantly improve the outcome of a graft in RDEB patients in terms of resistance and mechanical properties. Furthermore, if the *COL7A1* gene was introduced in fibroblasts as well as in keratinocytes, COLVII production at the DEJ should be optimal. A variety of methods, based on the addition of biomaterials, synthetic materials or exogenous extracellular matrix, are proposed to produce bilayered skin substitutes (Bell *et al.*, 1979; Berthod *et al.*, 1993; Boyce *et al.*, 2006; Sun *et al.*, 2014). A bilayered tissue-engineered skin (TES) was produced by the self-assembly approach, in which the cells produce and organise their own extracellular matrix (Larouche *et al.*, 2016; Michel *et al.*, 1996; Pouliot *et al.*, 2002). Autologous TESs are successfully used to treat ulcers and burn patients (Duranceau, 2014). A clinical trial assessing the efficacy of autologous TESs for the permanent coverage of full-thickness wounds is currently ongoing in Canada (ClinicalTrials.gov; NCT02350205).

In this study, the use of retroviral vectors produced from the 293Vec packaging cell lines was investigated for their ability to transduce fibroblasts and keratinocytes in presence of the EF-C peptide. The potential of transduced cells for producing and organising COLVII at the DEJ of TESs produced by the self-assembly approach was also assessed for the first time.

Materials and Methods

Ethics

The study was approved by the Ethics committee of the CHU de Québec - Université Laval Research Centre, Quebec City, Quebec for the protection of human subjects and conducted in accordance with the Helsinki Declaration of 1975.

Plasmids

The GFP retroviral plasmid was derived from GFP3 and contained a hygromycin resistance gene (Hygror) followed by an encephalomyocarditis virus internal ribosomal entry site sequence linked to the *GFP* gene. Both genes were under the control of the Moloney murine leukaemia virus (MoMLV) long terminal repeat (LTR) sequences (Qiao *et al.*, 2002). The *pLZRS-HACOL7A1* retroviral plasmid, containing a human influenza haemagglutinin (HA)-tagged full-length human *COL7A1* gene under the control of the MoMLV LTR sequence, was kindly provided by Alfred Lane (Stanford University School of Medicine, Standford, CA, USA) (Siprashvili *et al.*, 2010).

Cell culture

HT-1080 cells, 293T cells, the retrovirus parental packaging cell lines 293Vec-Ampho, 293Vec-Baev,

293Vec-Galv, 293Vec-RD114 (Ghani *et al.*, 2007; Ghani *et al.*, 2009) and their derivatives, containing the GFP vector and the HA-COL7A1 vector, were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Burlington, ON, Canada) supplemented with 10 % foetal calf serum (Wisent Bioproducts Inc, Saint-Jean-Baptiste, QC, Canada) and 1 % penicillin (Sigma-Aldrich, St Louis, MO, USA)/gentamicin (Galenova, St-Hyacinthe, QC, Canada). The retrovirus-producer cell lines were generated by infection with VSV-G pseudotyped recombinant retroviruses produced by transient transfection of 293T cells. Subsequently, producer cells infected with the GFP virus were selected in 200 µg/mL hygromycin (Calbiochem, Etobicoke, ON, Canada), resulting 99 % GFP positive, as assessed by fluorescence-activated cell sorting (FACS) analysis. The 293Vec-Ampho-HA-COL7A1 cells were assessed by immunofluorescence.

Human fibroblasts (18- and 37-year-old donors) and keratinocytes (26- and 37-year-old donors) were obtained from reductive breast surgery of healthy adult women after informed consent was given. Fibroblasts and keratinocytes were isolated by the two-step thermolysine and trypsin method, as previously described (Germain *et al.*, 1993; Lavoie *et al.*, 2013). Briefly, skin biopsies were digested overnight at 4 °C in HEPES buffer [10 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (MP Biomedicals Inc., Montreal, QC, Canada), 6.7 mM KCl, 142 mM NaCl and 1 mM CaCl₂] containing 500 mg/mL thermolysine (Sigma-Aldrich). Then, the dermis was separated from the epidermis with the use of forceps. Keratinocytes were dissociated from the epidermis for 15 min at 37 °C in a trypsin/ethylene-diaminetetraacetic acid (EDTA) solution [0.05 % trypsin 1 : 500 (Intergen, Toronto, ON, Canada) and 0.01 % EDTA/disodium salt prepared in phosphate-buffered saline (PBS)]. Next, fibroblasts and keratinocytes were collected by centrifugation and plated for primary cell culture (P0) and further passages. Cells were cryopreserved by the slow-rate cooling method, where primary cultures of freshly extracted keratinocytes or fibroblasts are trypsinised, counted and centrifuged. Cell pellets were kept on ice until resuspension at a density of 1 × 10⁶ or 2 × 10⁶ cells/mL in a cold (4 °C), freezing medium consisting of 90 % foetal calf serum and 10 % dimethyl sulphoxide (DMSO; Sigma-Aldrich) and, then, distributed in cryogenic vials (Nalgene® Labware, Rochester, NY, USA). Next, cryogenic vials were placed in a freezing container (Nalgene® Labware) filled with 99 % ethanol and precooled at 4 °C and kept at -80 °C for 24 h to allow a freezing rate of -1 °C/min. Cells were stored in liquid nitrogen until use (Germain *et al.*, 1993; Lavoie *et al.*, 2013).

Fibroblasts were thawed at 37 °C and cultured at passages three to four (P3-4) in DMEM containing 10 % foetal calf serum and antibiotics. Keratinocytes were thawed at 37 °C and cultured at passage one (P1) in keratinocyte medium [3 : 1 mixture of the

DMEM and Ham's F12 medium (Life Technologies)], supplemented with 5 % FetalClone II (HyClone™, Logan, UT, USA), 5 µg/mL insulin (Sigma-Aldrich), 0.4 µg/mL hydrocortisone (Calbiochem), 0.212 µg/mL isoproterenol hydrochloride (Sandoz, Boucherville, QC, Canada), 10 ng/mL epidermal growth factor (Austral Biologicals, San Ramon, CA, USA), 100 IU/mL penicillin G and 25 µg/mL gentamicin (Sigma-Aldrich). For all experiments, keratinocytes were co-cultured with irradiated human fibroblast feeder layers (iHFL), as previously described (Bisson *et al.*, 2013). Briefly, iHFL were pre-seeded in flasks at a concentration of 8000 cells/cm² at least one week prior to use and kept in culture for a maximum of one month. Keratinocytes in every condition were passaged when they reached 80-90 % confluence (Bisson *et al.*, 2013).

Retroviral infection

GFP viral titers were determined by scoring positive target cells by FACS analysis. Briefly, HT-1080 cells were inoculated at a density of 1 × 10⁵ cells/well in 24-well plates and cultured overnight in 1 mL of medium. The medium from each well was replaced with serial dilutions of virus supernatant in 1 mL final volume containing 8 µg/mL hexadimethrine bromide (polybrene; Sigma-Aldrich). 48 h later, cells were trypsinised and analysed for GFP fluorescence by FACS. For the HA-COL7A1 vector, HT-1080 cells were infected in 6-well plates and positive cells for HA were measured by immunofluorescence. Vector titers were calculated as follows: titer = F × Cinf/V × D, where F was the percentage of GFP- or HA-positive cells, determined by flow cytometry or immunofluorescence; Cinf the total number of target cells at the time of infection; V the viral volume applied; D the virus dilution factor. Infections resulting in 2-20 % of GFP- or HA-positive cells were considered for titer calculation based on the linear range of the assay. Titers obtained for the four pseudotyped GFP viruses were 2 × 10⁷ infectious viral particles (ivp)/mL. The titer for the 293Vec-Ampho-HA-COL7A1 was 2 × 10⁵ ivp/mL.

The infectivity of the four GFP pseudotyped retroviruses in presence of polybrene and EF-C (Genscript, Piscataway Township, NJ, USA) was assessed by infecting normal fibroblasts or keratinocytes plated at a density of 13,000 cells/cm² in 12-well plates. Fibroblasts were incubated for 2 h and keratinocytes for 4 h at 37 °C in 8 % CO₂. Then, fibroblasts were transduced at a multiplicity of infection (MOI) of 0.3 and keratinocytes at MOI of 1 in the presence of 8 µg/mL of polybrene or 10 µg/mL of EF-C. Transduction efficiency was evaluated by FACS analysis. The transduction with the HA-COL7A1 vector was performed similarly to the GFP viruses, but in 75 cm² flask at MOI of 0.36 only for fibroblasts (18-year old donor) and keratinocytes (26-year old donor). The expression of HA-COL7A1 was assessed by immunofluorescence.

Colony-forming efficiency assay

For determination of colony-forming efficiency (CFE), 1×10^2 keratinocytes/cm² were plated in Petri dishes with iHFL (8×10^3 cells/cm²) and maintained for 9 d in monolayer cultures (Barrandon and Green, 1987; Lavoie *et al.*, 2013; Rheinwald and Green, 1975). Three Petri dishes per condition were seeded. Then, colonies were fixed in 3.7 % buffered formaldehyde and analysed using a fluorescent imaging scanner (Typhoon TRIO+ Variable Mode Imager; GE Healthcare Life Sciences, Chicago, IL, USA). Using an inverted microscope (Zeiss Axio Observer.A1, Zeiss, Oberkochen, Germany), colonies were classified as

holoclones, meroclones and paraclones, according to the size of colonies and the morphology of cells forming them, as described by Barrandon and Green (1987). The percentage of CFE was calculated as follows: number of holoclones, meroclones and paraclones formed divided by the number of seeded cells multiplied by 100. The percentages of GFP-positive holoclones and meroclones were compared among the different conditions.

Tissue-engineered skin substitutes

The tissue-engineered skin method used derived from the self-assembled skin substitute method referred

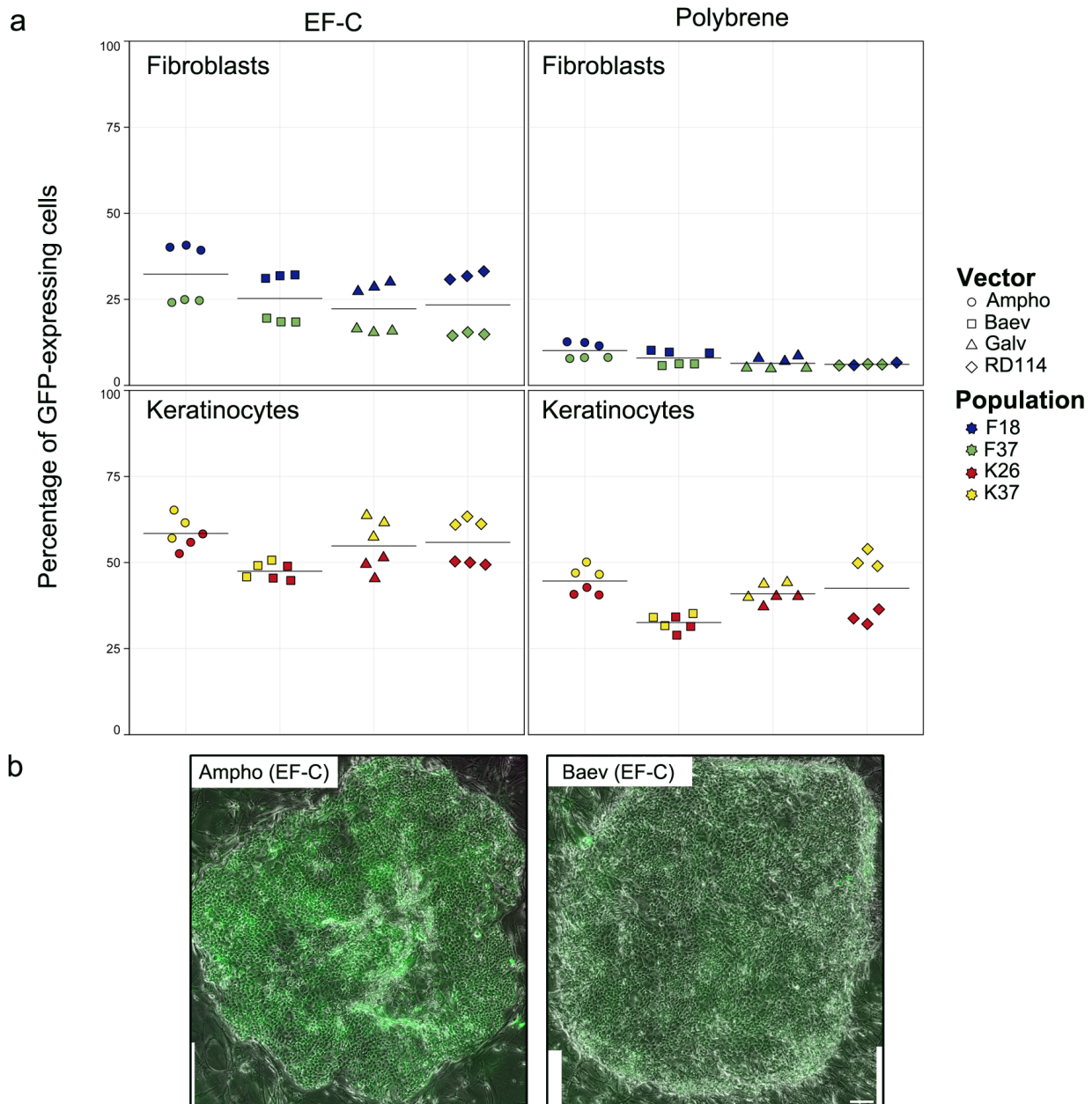


Fig. 1. (a) Analysis of GFP-expressing cells by flow cytometry. Effect of pseudotyped particles Ampho, Baev, Galv and RD114 on transduction of fibroblasts at MOI 0.3 and keratinocytes at MOI 1. Effect of the enhancers polybrene and EF-C peptide on transduction of fibroblasts and keratinocytes. (b) Representative images of GFP-positive holoclones. For the presented colonies, viral particles pseudotyped with Ampho (left panel) and Baev (right panel) were used to transduce cells in presence of the EF-C peptide. F18, fibroblasts from an 18-year old donor; F37, fibroblasts from a 37-year old donor; K26, keratinocytes from a 26-year old donor; K37, keratinocytes from a 37-year old donor. Scale bar: 200 μ m.

Table 1. Colony-forming efficiency assay. Ampho, amphotropic; Baev, baboon endogenous virus; CFE, colony-forming efficiency; Galv, gibbon ape leukaemia virus; GFP, green fluorescent protein; RD114, feline endogenous retrovirus; $n = 3$ (sample size); $N = 1$ (cell population size).

	Colony forming efficiency	Percentage of GFP-positive holoclones and meroclones
	Mean (SD)	Mean (SD)
Untransduced	14.63 % (1.10)	-
Untransduced		
EF-C	12.80 % (3.05)	-
Polybrene	12.17 % (1.34)	-
Ampho		
EF-C	5.50 % (1.30)	10.83 % (2.83)
Polybrene	4.97 % (0.86)	3.87 % (2.42)
Baev		
EF-C	6.07 % (0.15)	5.28 % (1.53)
Polybrene	5.97 % (0.32)	5.07 % (4.53)
Galv		
EF-C	5.57 % (0.38)	7.99 % (2.95)
Polybrene	6.73 % (1.00)	3.60 % (3.26)
RD114		
EF-C	6.60 % (0.79)	6.74 % (1.67)
Polybrene	8.30 % (0.30)	1.56 % (2.71)

to as “SASS-4” in Larouche *et al.* (2016). TESs were produced with different combinations of transduced cells at MOI of 0.36 and untransduced fibroblasts and keratinocytes. Briefly, 12,000 fibroblasts/cm² at P5, transduced or not, were cultured for 26 d in a fibroblast medium supplemented with 50 µg/mL ascorbic acid (Sigma-Aldrich), until the production of a fibroblast-derived tissue sheet. P2 keratinocytes, transduced or not, were seeded at a density of 200,000 cells/cm² on one of the two fibroblast-derived tissue sheets and submerged for 4 d in the keratinocyte medium supplemented with 50 µg/mL ascorbic acid. Then, keratinocytes/fibroblasts sheets were superimposed over a fibroblast-derived tissue sheet to produce the TES. TESs were cultured for 21 d at an air-liquid interface in keratinocyte medium containing 50 µg/mL ascorbic acid and without epidermal growth factor.

Fluorescence-activated cell sorting, immunofluorescence and histologic analysis

GFP expression in transduced cultured cells was assessed by FACS analysis. HT-1080 cells, fibroblasts and keratinocytes were trypsinised 72 h after infection and analysed for GFP expression. Fluorescence was measured with a Coulter EPICS XL-MCL flow cytometer and Expo32 software (Beckman Coulter, Brea, CA, USA) or with a FACSCalibur™ (Becton Dickinson, San Jose, CA, USA).

HA-COLVII expression in transduced cells and in retrovirus-producer cells was assessed by immunofluorescence. HT-1080 cells, 293Vec-Ampho-HA-COL7A1 cells, keratinocytes and fibroblasts were cultured in 6-well plates until they reached sub-confluence. Cells were fixed with methanol

(ThermoFisher Scientific, Waltham, MA, USA) or 2 % paraformaldehyde followed by 0.2 % Triton X-100 (BioRad, Mississauga, ON, Canada). Next, cells were incubated with a primary mouse monoclonal antibody raised against HA (1 : 500, clone HA.11; Biolegend, San Diego, CA, USA) followed by an Alexa488-conjugated goat anti-mouse (1 : 1000; ThermoFisher Scientific); nuclei were coloured with Hoechst reagent 33258 (Sigma-Aldrich). The number of transduced cells expressing the HA tag was scored by image analysis of at least 20 randomly selected fields. The number of HA-positive cells was divided by the number of cell nuclei.

For immunofluorescence analyses, biopsies from TESs were embedded in Tissue-Tek OCT Compound (Somagen, Edmonton, AB, Canada) and frozen in liquid nitrogen. Immunofluorescence assays for transgene expression and markers were performed on 5 mm-thick cryosections, permeabilised with cold acetone, using the following antibodies: mouse monoclonal anti-HA (1 : 500; clone HA.11, Biolegend), rabbit polyclonal anti-human collagen IV (1 : 400; Abcam, Cambridge, MA, USA), mouse monoclonal anti-K10 (1 : 200; clone RKSE60; Cedarlane, Burlington, ON, Canada), rabbit polyclonal anti-K14 (1 : 600; Cedarlane), mouse anti-human Ki67 (1 : 800; Becton Dickinson, Franklin Lakes, NJ, USA), rabbit anti-human loricrin (1 : 240; Covance, Dedham, MA, USA) and mouse anti-human K19 clone A53-B/A244 [1 : 200; gift from U. Karsten, Institute of Biological Sciences, University of Rostock, Germany (Kasper *et al.*, 1987)]. The following secondary antibodies were used: Alexa-488-conjugated goat anti-mouse (1 : 1000) and Alexa-594-conjugated goat anti-rabbit (1 : 500) (ThermoFisher Scientific).

For histological analyses, biopsies from TESs were fixed in Histochoice (Amresco, Solon, OH, USA) and embedded in paraffin. 5 mm-thick microtome sections were stained with haematoxylin and eosin.

Results

Efficient transduction of human fibroblasts and keratinocytes with vectors produced from 293Vec packaging cell lines in presence of the EF-C peptide

Retroviral vectors pseudotyped with the RD114 and Galv envelopes are highly efficient to transduce cells from haematopoietic origin (Ghani *et al.*, 2009; Kelly *et al.*, 2000; Kiem *et al.*, 1997). Our first objective was to discriminate which envelope among Ampho, Baev, Galv and RD114 was the most efficient for the transduction of human fibroblasts and keratinocytes. In addition, the efficiency of polybrene and the EF-C peptide to enhance transduction was compared. GFP was used as a reporter gene to evaluate the retroviral-mediated gene transfer efficacy. Fibroblasts and keratinocytes, extracted from two different healthy donors, were tested. For the two fibroblast populations transduced in the presence of polybrene, the average percentage of GFP-positive fibroblasts was 10.1 % with the Ampho vector and 7.9 %, 6.4 % and 6.1 % with the Baev, Galv and RD114 viruses, respectively (Fig. 1a). In the presence of the EF-C peptide, 32.3 % of fibroblasts were transduced with the Ampho vector as compared to 25.3 %, 22.3 % and 23.4 % with the Baev, Galv and RD114 viruses, respectively (Fig. 1a). A similar trend was observed for the two tested keratinocyte populations: the Ampho pseudotyped vector was slightly more efficient than the other vectors. In presence of polybrene, the average percentage of GFP-positive keratinocytes was 44.6 % with the Ampho vector as compared to 32.6 %, 40.9 % and 42.5 % with the Baev,

Galv and RD114 vectors, respectively. In presence of the EF-C peptide, 58.4 % of keratinocytes were GFP-positive with the Ampho vector, compared to 47.5 %, 54.8 % and 55.9 % with the Baev, Galv and RD114 vectors, respectively (Fig. 1a). Next, the effect of the transduction enhancers and vectors on clonogenic behaviour of keratinocytes was evaluated. The percentage of colonies formed is indicated in Table 1 and it was in line with previous studies (Barrandon and Green, 1987; Lavoie *et al.*, 2013; Rheinwald and Green, 1975). The transduction enhancers did not appear to affect the CFE. However, regardless of the vector used, a decrease in CFE was observed for transduced, compared with untransduced, keratinocytes (Table 1). The different vectors used did not appear to affect the proportion of GFP-expressing colonies of transduced keratinocytes (Table 1). Also, the morphology of GFP-expressing colonies was evaluated. Typically, keratinocyte stem cells form large colonies, mainly composed of small cells called holoclones (Barrandon and Green, 1987). For each vector, some GFP-expressing holoclones were observed (Fig. 1b), indicating that the transduction protocol allowed for the preservation of keratinocyte stem cells.

Considering that for both keratinocytes and fibroblasts, the vector produced from the Ampho packaging cell line was the most efficient to transfer the GFP gene, while maintaining a normal cellular morphology, experiments were pursued using this packaging cell line. Also, in human fibroblasts and keratinocytes, the EF-C peptide outperformed polybrene as a transduction enhancer of the retroviral-mediated gene transfer.

Efficient retroviral delivery of the COL7A1 gene to human fibroblasts and keratinocytes in the presence of EF-C

The next step was to produce fibroblast and keratinocyte populations that stably expressed an exogenous form of COLVII. The COL7A1 cDNA, delivered by the vector, was linked to a HA tag to allow the discrimination between the endogenous COLVII present in healthy donor cells and the protein expressed by the transgene. To generate stable retrovirus-producer cells, 293Vec-Ampho cells were infected with a HA-COL7A1 virus. Then, cells were cultured until they reached confluence, when the viral supernatant was harvested. 19 % of these cells were HA-positive [standard deviation (SD) = 4, $n = 34$ (sample size), $N = 1$ (cell population size)] (Fig. 2). Subsequently, fibroblasts and keratinocytes were infected and the expression of the transgene was assessed by immunofluorescence (Fig. 3). As observed by flow cytometry for the GFP expression, the percentage of transduction of both fibroblasts and keratinocytes was higher when EF-C was used, compared with polybrene. Indeed, 19 % of fibroblasts were transduced (SD = 5, $n = 19$, $N = 1$) in the presence of EF-C and only 4 % with polybrene (SD = 2,

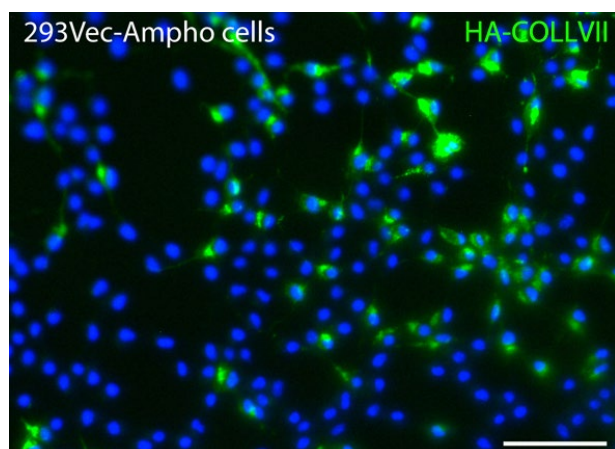


Fig. 2. Representative picture of 293Vec-Ampho cells transduced with the HA-COL7A1 vector immunolabeled with anti-HA antibody (green). Hoechst staining of cell nuclei in blue. HA, haemagglutinin tag. Scale bar: 100 μm .

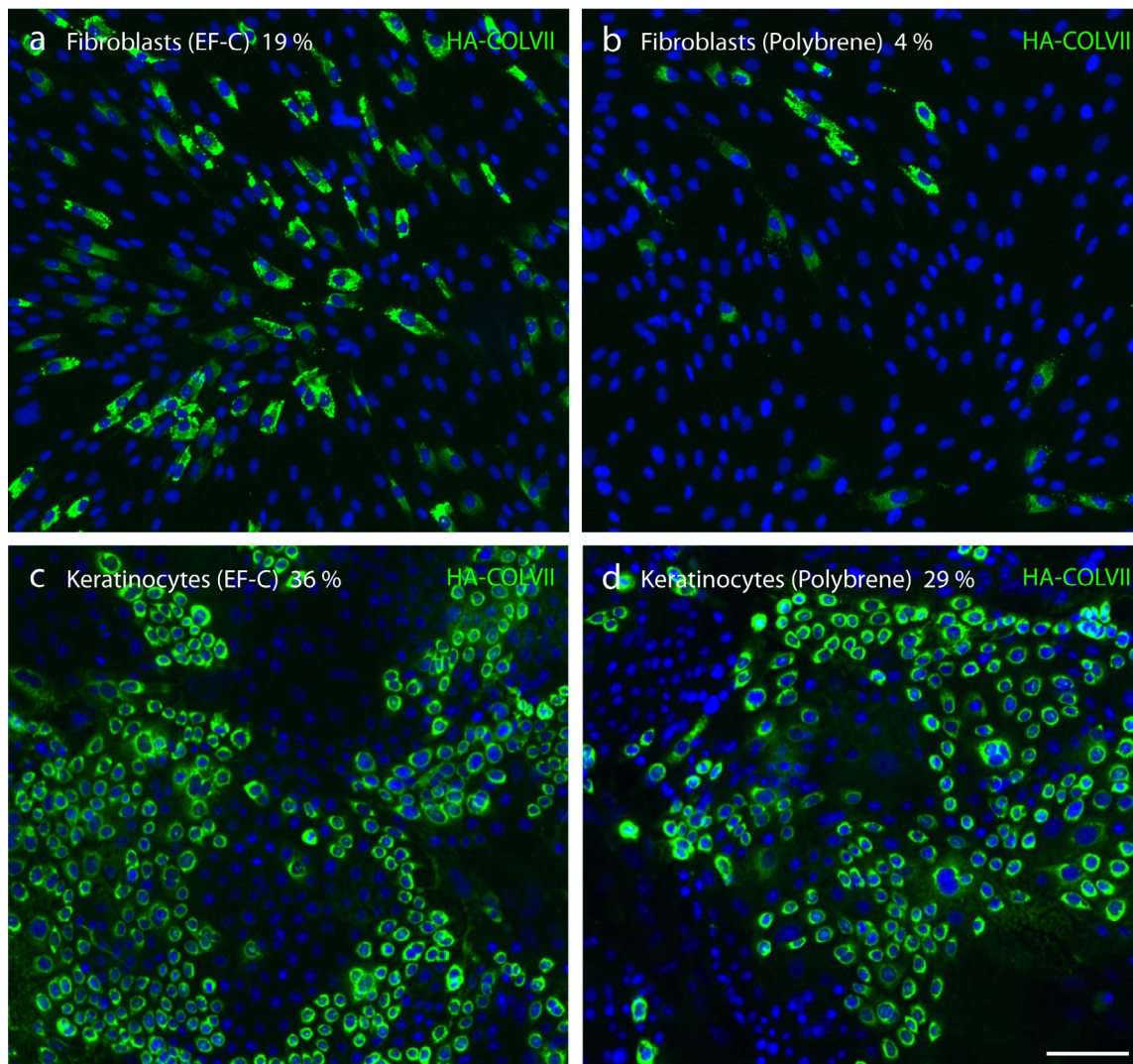


Fig. 3. Expression of HA-COLVII in monocultured transduced cells. Wild-type fibroblasts transduced with the *HA-COL7A1* vector produced from the 293Vec-Ampho cells in the presence of (a) EF-C or (b) polybrene. Wild-type keratinocytes transduced with the *HA-COL7A1* vector produced from the 293Vec-Ampho cells in the presence of (c) EF-C or (d) polybrene. Immunolabelling with anti-HA antibody in green. Hoechst staining of cell nuclei in blue. Scale bar: 100 μ m.

$n = 20$, $N = 1$). The difference was less striking with keratinocytes: 36 % (SD = 6, $n = 20$, $N = 1$) of cells were infected in the presence of EF-C compared to 29 % (SD = 4, $n = 20$, $N = 1$) with polybrene. These results indicated that the viral delivery of the *COL7A1* gene to human fibroblasts and keratinocytes was higher when EF-C was used.

Transduced fibroblasts and keratinocytes in tissue-engineered skin substitutes expressed exogenous COLVII

TESs were produced with untransduced cells or cells transduced in the presence of either EF-C or polybrene. Then, different cell combinations were tested: transduced fibroblasts and transduced keratinocytes (F+/K+ TES); transduced fibroblasts and untransduced keratinocytes (F+/K- TES); untransduced fibroblasts and transduced keratinocyte (F-/K+ TES) and control untransduced fibroblasts and untransduced keratinocytes (F-/K- TES). HA-

COLVII was detected at the DEJ in F+/K+ TESs, F+/K- TESs and F-/K+ TESs (Fig. 4) and it colocalised with collagen IV, an extracellular matrix protein that is normally present at the DEJ. A continuous intense deposit of HA-COLVII was observed in the F+/K+ TESs transduced in the presence of the EF-C peptide (Fig. 4a). The staining was continuous, but less intense in F+/K- TES (Fig. 4b) or F-/K+ TESs (Fig. 4c). When the transductions were made in the presence of polybrene, the HA-COLVII staining at the DEJ of F+/K+ TESs, F+/K- TESs and F-/K+ TES was discontinued and less intense in comparison with their counterparts transduced in the presence of the EF-C peptide (Fig. 4d-f). The expression of HA-COLVII was also detected in the cytoplasm of keratinocytes in all epidermal layers, as previously observed by others and explainable by the ubiquitous expression of the viral promoter LTR (Gache *et al.*, 2004; Sipsrshvili *et al.*, 2010). HA-COLVII was undetected in control F-/K- TESs (Fig. 4g).

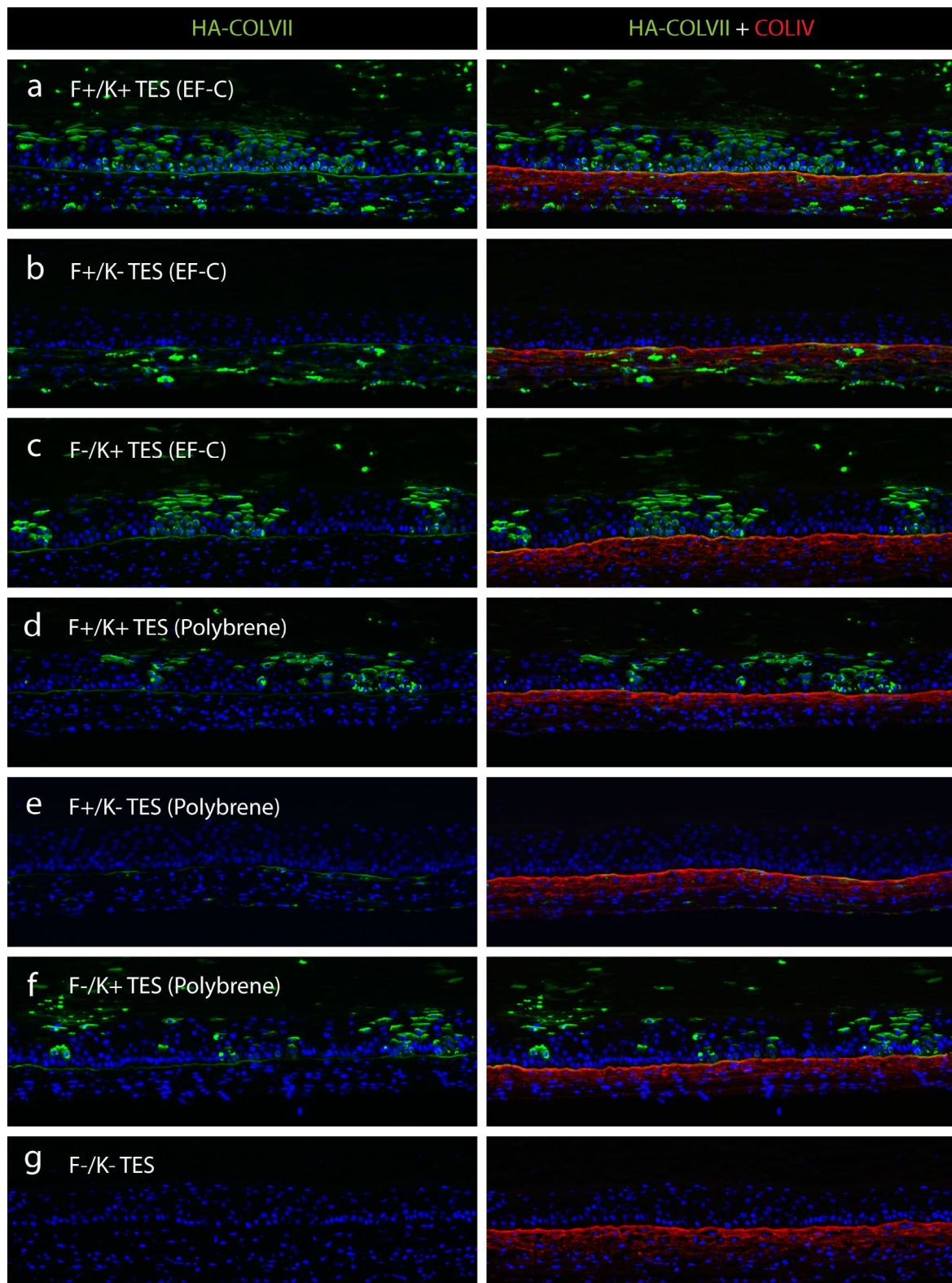


Fig. 4. Production of HA-COLVII in tissue-engineered skin substitutes cultured in the air-liquid interface for 21 d. Detection of HA-COLVII (left and right columns, green) and type IV collagen (right column, red) by immunofluorescence staining. (a,d) F+/K+ TESs, (b,e) F+/K- TESs, (c,f) F-/K+ TESs and (g) controls F-/K- TESs. Cells were transduced in the presence of (a-c) EF-C and (d-f) polybrene. Hoechst staining of cell nuclei in blue. F- and K- indicate untransduced fibroblasts and keratinocytes, respectively. F-/K- indicates control tissue-engineered skin made from untransduced fibroblasts and untransduced keratinocytes. F+ and K+ indicate transduced fibroblasts and keratinocytes, respectively. F+/K+ TES, F-/K+ TES, F+/K- TES, indicate the 3 types of tissue-engineered skin made from transduced fibroblasts and/or transduced keratinocytes. COLIV, type IV collagen; HA-COLVII, haemagglutinin-tagged type VII collagen. Scale bar: 100 μm .

Transduced keratinocyte stem cells were preserved in tissue-engineered skin substitutes

Long-term expression of a transgene in keratinocytes is only possible if stem cells are transduced. For this reason, the concomitant expression of HA-COLVII with keratin 19 (K19), which is expressed by keratinocyte stem cells (Lavoie *et al.*, 2013; Michel *et al.*, 1996; Pontiggia *et al.*, 2009), was investigated. With both transduction enhancers, HA-COLVII expression was detected in K19-positive basal keratinocytes of the TES epidermis (Fig. 5). This result suggested that the transduction protocol used allowed for the transduction of keratinocyte stem cells.

The epidermal proliferation and differentiation profiles were conserved in tissue-engineered skin substitutes made of transduced cells

Normal proliferation and differentiation of the TES epidermis are required for the maintenance of the skin homeostasis. The expression of proliferation and epidermal differentiation markers was assessed by immunofluorescence assays and the results showed a similar expression pattern in TESs produced with

transduced cells (F+/K+ TESs, F+/K- TESs and F-/K+ TESs) as compared with control F-/K- TESs produced with untransduced cells (Fig. 6). The basal keratinocytes expressed keratin 14 (K14) and the proliferation marker Ki67 was detected in the nucleus of many cells within the basal layer. Keratinocytes in suprabasal layers expressed the differentiation marker keratin 10 (K10), whereas loricrin, a granular-layer marker, was present in higher suprabasal layers (Fig. 6a-f).

Haematoxylin and eosin histological analyses of TESs produced with transduced or untransduced cells revealed well-organised skin-like tissues (Fig. 6g-i). These results indicated that the transduction process did not alter the histological integrity of TESs.

Discussion

In this study, an efficient method for retroviral gene transfer into human fibroblasts and keratinocytes using the EF-C peptide was described. Also, TESs produced from transduced fibroblasts and

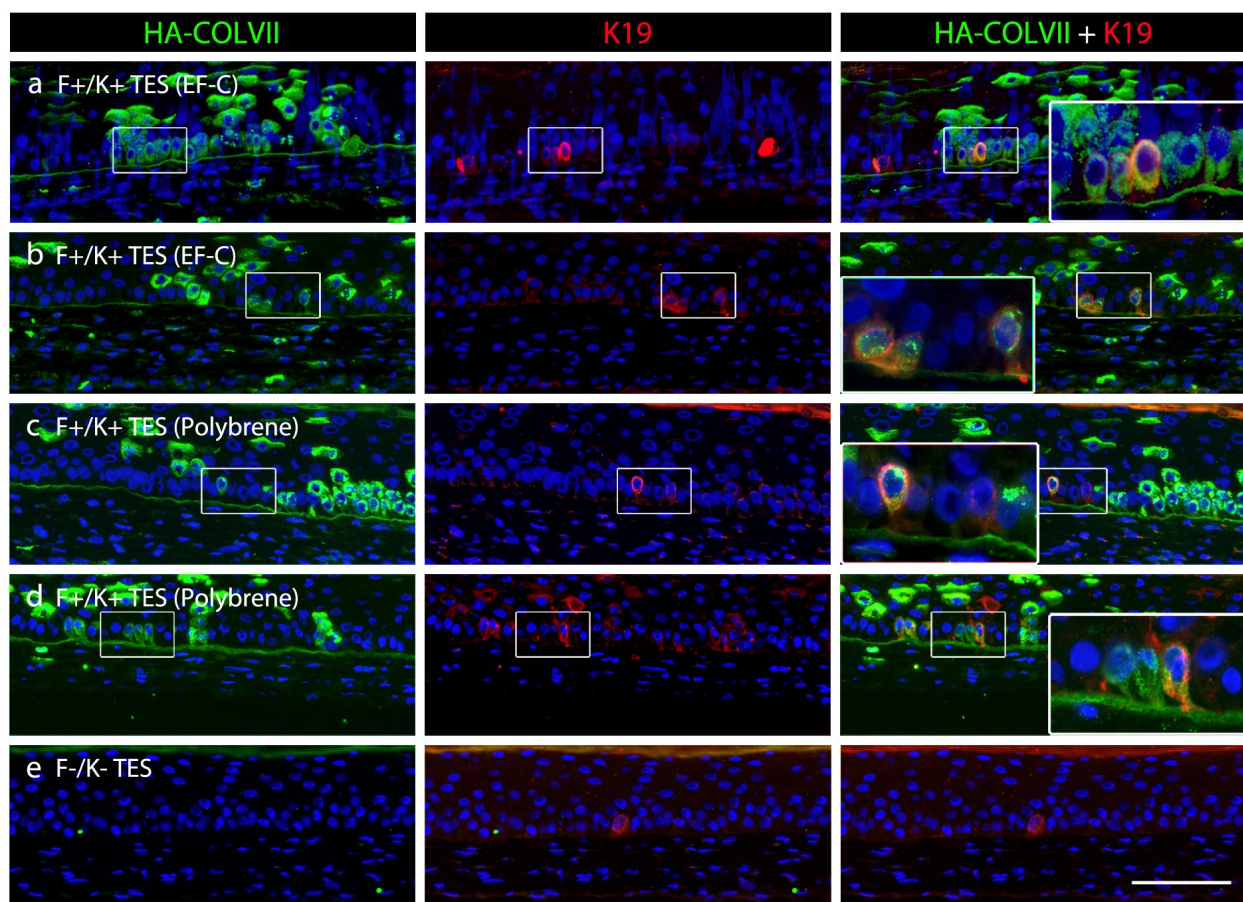


Fig. 5. Co-detection of HA-COLVII and K19 in tissue-engineered skin substitutes cultured 21 d at the air-liquid interface. F+/K+ TESs transduced in the presence of (a,b) EF-C and (c-d) polybrene. (e) Control F-/K- TES. Immunolabelling of HA-COLVII with anti-HA antibody (green) and anti-K19 (red) is shown in single labelling (left and centre columns) and double (right column). An enlargement of the boxed areas with double positive cells is shown as inserts in the right column. Hoechst staining of cell nuclei in blue. Control F-/K- TESs, tissue-engineered skin made from untransduced fibroblasts and untransduced keratinocytes; F+/K+ TES, tissue-engineered skin made from transduced fibroblasts and transduced keratinocytes. Scale bar: 100 μ m.

keratinocytes expressing the COLVII protein, resulting from the inserted gene, were generated. HA-COLVII expression in K19-positive basal keratinocytes and the presence of transduced holoclones suggested that keratinocyte stem cells were successfully transduced, allowing for the expression of the inserted gene over time. A continuous deposit of the exogenous COLVII was present at the DEJ of TESs, with the best results obtained with cells transduced in presence of the EF-C peptide.

The low transduction efficiency of the target cells is a limiting factor for gene therapy applications that can be improved by different means. In the case of retroviral gene delivery, new versions of stable retrovirus packaging cell lines have been developed. These cells are safer and release recombinant retroviruses with higher titers compared with former versions (Cosset *et al.*, 1995; Sheridan *et al.*, 2000). The GFP viruses used in this study were produced with a set of 293-based retrovirus packaging cell lines, allowing for the production of vectors with titers above 10^7 ivp/mL (Ghani *et al.*, 2007; Ghani *et al.*, 2009). The transduction ability of GFP vectors

pseudotyped with the Ampho, Baev, Galv and RD114 envelopes was assessed. It was found that the Ampho envelope was more efficient to transduce GFP into fibroblasts than Baev, Galv and RD114.

The long size of the *COL7A1* cDNA hinders the generation of high-titer vectors (Goto *et al.*, 2006; Siprashvili *et al.*, 2010; Titeux *et al.*, 2010). In our study, the titer obtained with the *HA-COL7A1* vector from the 293Vec-Ampho population was 2×10^5 ivp/mL. As only 19 % of the 293Vec cells were *HA-COL7A1*-positive (Fig. 2), selection of isolated retrovirus-producer clones could be expected to result in titers higher than 10^6 ivp/mL. However, a retrovirus-producer clone was intentionally not selected, as the *HA-COL7A1* vector was not planned to be used for clinical applications. Retrovirus-producer clones, with a codon-optimised version of the *COL7A1* cDNA in a self-inactivated (SIN) retroviral backbone, are currently under development in our laboratory and by others using our 293Vec cells (Hennig *et al.*, 2014). Codon optimisation is used to enhance the stability of the transcript, aiming at obtaining high viral titers and transgene expression in transduced cells

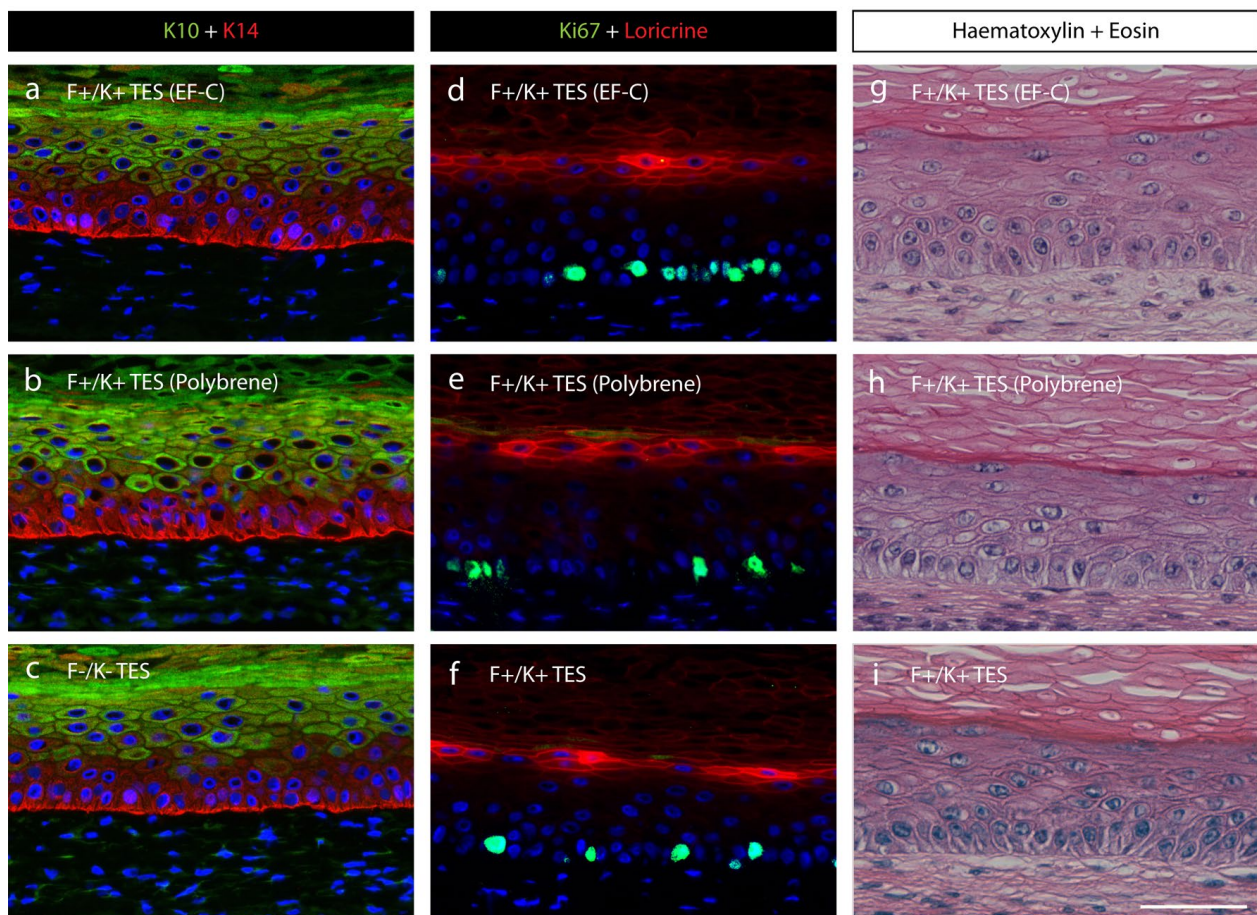


Fig. 6. Differentiation profile of tissue-engineered skin substitutes cultured 21 d at the air-liquid interface. (a-c) Double immunodetection of K10 (green) and K14 (red). (d-f) Double immunodetection of the proliferation marker Ki67 (green) and the late differentiation marker loricrin (red). Hoechst staining of cell nuclei in blue. (g,i) Histological analysis of TESs. F+/K+ TES transduced in the presence of (a,d,g) EF-C and (b,e,h) polybrene; (c,f,i) control F-/K- TES. F-/K- TESs, tissue-engineered skin made from untransduced fibroblasts and untransduced keratinocytes; F+/K+ TES, tissue-engineered skin made from transduced fibroblasts and transduced keratinocytes; K10, keratin 10; K14, keratin 14. Scale bar: 100 μ m.

(Moreno-Carranza *et al.*, 2009). Also, SIN retroviral vectors without viral enhancers are safer and less prone to transformation upon integration when compared to vectors with intact LTRs (Cavazza *et al.*, 2013; Hacein-Bey-Abina *et al.*, 2008; Howe *et al.*, 2008; Modlich *et al.*, 2006). Fibronectin and retronectin enhance retroviral gene transfer in keratinocytes (Bajaj *et al.*, 2005; Goto *et al.*, 2006), but the cost of the good manufacturing practices (GMP)-grade fibronectin or retronectin and the numerous manipulation steps limit the clinical development of a gene therapy strategy for RDEB. One alternative could be the use of a molecule easier to manipulate than retronectin or fibronectin that could be added simultaneously with the vector, without further manipulations. The EF-C peptide could be such a molecule as it has the ability to self-assemble into nanofibrils and to increase retroviral transduction of peripheral blood lymphocytes, macrophages and haematopoietic stem cells (Yolamanova *et al.*, 2013). In the current study, it was found that, in fibroblasts, EF-C increased the gene transfer of *GFP* and *HA-COL7A1* vectors by 3.1-fold and 4.5-fold, respectively (Fig. 1a), when compared to polybrene (Fig. 3c,d). A 1.3-fold increase in keratinocyte transduction was obtained with EF-C, but, as the infection values were high and not in the linear range, the difference was likely more than 2-fold.

A sustained expression of COLVII in grafted skin for the whole patient's life is desirable for an effective gene therapy treatment of RDEB. A recent paper from De Luca's group shows that the grafting of transfected holoclones over 80 % of total body surface area results in long-term correction in a patient suffering from JEB (Hirsch *et al.*, 2017). As described in our study for RDEB cells (Fig. 4), and by others (Georgiadis *et al.*, 2016; Goto *et al.*, 2006; Jackow *et al.*, 2016; Titeux *et al.*, 2010), transduced fibroblasts produced COLVII that relocated at the DEJ. However, the long-term persistence of these cells in our TESs remains to be proven. As shown previously, keratinocyte stem cells persist in the TES produced by the self-assembly approach and are suitable for the permanent closure of full-thickness skin injuries in human patients (Duranceau *et al.*, 2014). In the present study, transgene expression was observed in holoclones, which are associated to stem cells (Barrandon and Green, 1987). Moreover, the transgene expression persisted in the keratinocytes after several cell divisions occurring during the 28 d necessary between transfection and TES fixation. The presence of double positive keratinocytes for K19 and the transgene in the basal layer of the TES epidermis indicated that the *in vitro* model was adequate. The long-term stable expression of the transduced gene in TES *in vivo* remains to be confirmed after grafting in an animal model.

Siprashvili *et al.* (2016), in a gene therapy study with four RDEB patients treated with gene-corrected epidermal sheet grafts, report that COLVII expression

detected at the DEJ is associated with healing improvement, but the response is variable and declines over time (Siprashvili *et al.*, 2016). A failure to target keratinocyte stem cells, an induction of their differentiation during the gene-transfer procedure or a variable engraftment can explain these results. In this trial, the transduction efficiencies are in the range of 50-70 % with a spinoculation protocol (Siprashvili *et al.*, 2016). This percentage might have been insufficient to target stem cells and the harsh infection condition could have led to cellular differentiation. In comparison, more than 90 % of keratinocytes are transduced in the JEB studies with a gentle transduction procedure (De Rosa *et al.*, 2014; Hirsch *et al.*, 2017; Mavilio *et al.*, 2006). This important difference in viral transduction is due to the extended length of the *COL7A1* cDNA that prevents the generation of high-titer vector batches. The 3.5 kbp *LAMB3* cDNA used in the JEB studies leads to high-titer retroviral vector production.

In conclusion, our results showed that our optimised method to transfer gene in fibroblasts and keratinocytes allowed for the production of TESs that expressed and organised COLVII at the DEJ. Thus, it is believed that it is worth continuing the work on a TES genetically modified to correct RDEB, in order to develop a permanent local treatment for patients suffering from this disease.

Acknowledgments

Conflicts of interest: KG and MC are inventors on a patent (US2009098648) that has been licensed to BioVec Pharma. KG and MC own equity in BioVec Pharma.

All authors have made substantial contributions. Authors' contributions are as follow: ADP, DL, KG, FB, MC and LG conceived and designed the experiments. ADP, KG and FB performed the experiments and collected the data. ADP, DL, LG, SCG, SL, VJM and MC analysed the data. VJM, MC and LG provided reagents/materials/analysis tools. ADP, DL and MC wrote the paper. All authors revised the manuscript and approved the final version to be published. LG and MC are co-senior authors and acquired funding to perform the study.

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Editor's notes: All of the matters raised by the reviewers have been incorporated in the main text, so there is no Discussion with reviewers section for this paper.

The Scientific Editor responsible for this paper was Mauro Alini.