Supplementary Online Content

Siprashvili Z, Nguyen NT, Gorell ES, et al. Safety and wound outcomes following genetically corrected autologous epidermal grafts in patients with recessive dystrophic epidermolysis bullosa. *JAMA*. doi:10.1001/jama.2016.15588

This supplementary material has been provided by the authors to give readers additional information about their work.

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eMethods

Materials and reagents

All materials and reagents used during LEAES manufacturing were free of adventitionus viruses based on certification of analyses provided by manufacturer. Each lot tissue culture media that included bovine-derived reagents was tested via commercial 9CFR hemadsorption testing for adventitious viruses (American BioResearch, Pullman, WA) and found negative. Direct costs per patient were approximately $117K in hospital charges, supplies and reagents.

RDEB Keratinocyte isolation and expansion

The skin samples were obtained as two 8mm punch biopsies and transferred into 35 mL biopsy collection medium 50/50A (50% Keratinocyte Medium 154 with human keratinocytes growth supplement (Life Technologies, Carlsbad, CA) and 50% defined keratinocyte serum free media with supplement (Life Technologies, Carlsbad, CA)) with 30 µg/mL amikacin (HIkma Pharmaceuticals, London, United Kingdom), 20 µg/mL vancomycin (Sigma Aldrich, St. Louis, MO) and 0.5 µg/mL amphotericin B (USBiological, Salem, MA). To separate the epidermis from the dermis, the skin sample placed in dispase solution containing 25 caseinolytic units/mL of dispase (Life Technologies, Carlsbad, CA) for 16-20 hours at 5°C. The next day, epidermis was carefully peeled off the dermis and placed in TrypLE Select 10X (Life Technologies, Carlsbad, CA) solution at 37°C for 20-30 minutes. The solution was spun down at 1200 rpm to obtain a keratinocyte pellet. Cells were washed once with phosphate buffered saline (PBS, Life Technologies, Carlsbad, CA) and keratinocytes were plated on PureCoat Collagen I Mimetic Cultureware (Corning Life Sciences, Tewksbury, MA), in 50/50A media. After keratinocytes reached 60-70% confluence, cells were treated with TrypLE Select 10X and plated for viral transduction. At least $4x10^6$ cells were required to initiate the transduction process.

Keratinocyte correction

The cGMP grade GalV-pseudotyped LZRSE-COL7A1 virus containing full-length *COL7A1* cDNA under control of the MLV LTR was produced by Indiana University Vector Production Facility using current Good Manufacturing Practices as described by

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Siprashvili et al 2010.[1] Viral transduction was performed by overlaying 12 mL viral supernatant for each plate and centrifugation of cells at 1250 rpm and 32°C for 1 hour. After centrifugation, viral supernatant was removed by washing with PBS and 50/50V medium used for corrected keratinocyte expansion. Transduction was repeated as needed, as long as corrected KC continued to meet pre-release criteria of virus transduction efficiency (VTE) >50% and proviral genome copy number (PGCN) ≤ 3.

Pre-release testing:

VTE test

VTE test was performed using IF techniques with anti-type VII collagen monoclonal antibody NP32, NP185 or anti-type VII collagen polyclonal antibody FNC1. Cells were fixed in a solution of methanol/acetone mixture, permeabilized in detergent and incubated with anti-C7 primary antibody for 1 hr at room temperature. After extensive washes, secondary antibody conjugated to Alexa Fluor 555 dye (a red fluorescent antibody tag) was added and incubated for another 1 hr. Cell nuclei labeled with Hoechst 33342 for 10 minutes, washed, and mounted with prolong gold antifade reagent (Life Technologies, Carlsbad, CA). VTE was determined by counting the ratio of blue nuclei to C7 positive cells. To meet pre-release criteria, at least 50% of cells were positive for C7 expression.

PGCN test

PGCN test was performed via qPCR analysis of genomic DNA isolated from corrected RDEB KC post retroviral transduction. Genomic DNA was purified from $3x10^6$ corrected cells using Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germany). DNA was quantified using standard spectrophotometric techniques and used for qPCR analysis. Proviral dose was determined using threshold cycle (Ct) of the template and the standard curve of Ct dependence from the amount of plasmid DNA control. The average PGCN was calculated from: PGCN = (TPCN x 6.16pg) / ($C_{templ.}$ X 10^3)pg, where, TPCN = Total proviral copy number. 6.16pg = Amount of genomic DNA in the somatic cell. $C_{templ.}$ = Template amount used in PCR in nanograms. To meet pre-release criteria, no more than 3 proviral genome copy numbers were present on average for every keratinocyte genome.

Sterility test

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A sample of culture supernatant was tested for sterility by membrane filtration using the Millipore Steritest system, designed to eliminate potential false negatives from antibiotics present in the culture media (Pacific BioLabs, Hercules, CA). After filtration and washes, the sample was placed in Soybean Case in Digest Medium and Fluid Thioglycollate Medium and incubated for 14 days. Samples were observed for evidence of microbial contamination daily.

Endotoxin test

The sample of the cultured supernatant was assessed via the Limulus Amebocyte Lysate (LAL) QCL-1000 assay (Lonza, Basel, Switzerland) in accordance with manufacturer's recommendations. Results of this test were < 1.0 EU/mL in order to satisfy product release requirements.

Mycoplasma test

Cell culture supernatant was tested for mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland) in accordance with manufacturer's requirements. Results of this test were < 0.9 in order to satisfy product release requirements.

LEAES initiation and processing

Once corrected keratinocytes reached 100% confluence, the LEAES initiation process was started and growth medium was changed from 50/50V to epidermal sheet production medium DFF31, which consists of Dulbecco's Modified Eagle Medium (Life Technologies, Carlsbad, CA) and F12 medium (Lonza, Basel, Switzerland) containing 10% fetal bovine serum (Lonza, Basel, Switzerland), 36 ng/mL hydrocortisone (Spectrum, New Brunswick, NJ), 25 μg/mL adenine (Sigma Aldrich, St. Louis, MO), 5 μg/mL recombinant human Insulin (Sigma Aldrich, St. Louis, MO), 2 ng/mL liothyronine (Spectrum, New Brunswick, NJ), 5 μg/mL bovine transferrin (Millipore, Billerica, MA), 10 ng/mL recombinant epidermal growth factor (R&D Systems, Minneapolis, MN) and 30 μg/mL amikacin (Hikma Pharmaceuticals, London, United Kingdom), and 20 μg/mL vancomycin (Sigma Aldrich, St. Louis, MO).

LEAES assembly and transportation

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LEAES assembly was initiated the day of grafting. Epidermal sheets were released from the surface of the plate by enzymatic digestion with dispase (Life Technologies, Carlsbad, CA) for 20-30 min at 37°C. To remove residual amounts of medium and dispase, epidermal sheets were washed at least 5 times with 50/50VC. It was secured to the matched size petroleum gauze with surgical hemoclips and the basal side was marked with a sterile black suture. Assembled LEAES was submerged in 50/50VC transport medium and sealed with gas permeable sterile film. The LEAES epidermal grafts were then transported to the operating room for transplantation.

Release testing

Gram stain sterility test

On the day of LEAES release, a sample of culture media was sent to the Stanford Hospital Clinical Laboratory for a rapid gram stain test. A negative result of the test was used as a LEAES lot release criteria.

LEAES viability test

Viability testing was performed in which the LEAES sample was incubated with a nuclei dye mixture containing Hoechst 33342 and SYTOX Green stain for 20 minutes. The ratio of SYTOX green stain to Hoechst 33342 stain was calculated at ≥70% to release the product.

Post-release testing

Samples of the cultured medium and LEAES graft were submitted for send-out testing, with expecting test results to be obtained post graft transplantation due to a long duration of the testing process. A safety plan was in place in case these “post-release” test results was out of specification. Post-release criteria included additional sterility testing (see Sterility Test in pre-release testing), RCR testing (Indiana University Vector Production Facility) and mycoplasma testing (Bionique Testing Laboratories, Saranac Lake, NY).

RCR test

A sample of LEAES and LEAES cultured supernatant were patiented to the extended PG-4 S+L- cell plaque assay at Indiana University Vector Production Facility following their recommendations and result of the test “no evidence of RCR” used as release

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criteria. At baseline, 3 months, and 6 months, blood samples were analyzed by the Indiana University Vector Production Facility to determine the level of GALV envelope (GALV-E) sequences present using a quantitative polymerase chain reaction (Q-PCR). The adequacy of the amount of blood sample was assessed by a second probe and primer set for human apolipoprotein B gene sequences. A standard curve using genomic DNA containing 10^5, 10^4, 10^3, 10^2, and 10 copies of GALV-E sequence per 0.12ug of genomic DNA were used as a positive control. Negative controls consisted of untransduced human genomic DNA and water.

Cytotoxic T cell assay

15 mL of whole blood was collected at baseline, 1 month, 3 months and 6 months for the cytotoxic T cell assay. Peripheral blood mononuclear cells were isolated from buffy coats or whole blood using Ficoll-Paque (GE Healthcare) density-gradient centrifugation. Adherent monocytes were then recovered after a 2-hour incubation in Petri dishes. $CD4^+$ and $CD8^+$ T lymphocytes were purified together using MACS magnetic cell sorting kits (Miltenyi Biotech), by incubating the nonadherent cells with anti-CD4 and anti-CD8 antibodies conjugated to paramagnetic microbeads. A 96-well PVDF-filter plates (Millipore) were coated with monoclonal antibody against IFN-γ (BD Pharmingen) or IL-4 (BD Pharmingen), blocked using RPMI medium with 5% human AB serum, and washed with serum-free RPMI. $CD4^+$ and $CD8^+$ T lymphocytes (2×10^5 cells/well), and γ-irradiated monocytes (2.5×10^4 cells/well) were co-incubated on the plate in the presence of 20 UI/ml IL-2 for 40 hours at 37°C, in a humidified, 5% CO_2 in air incubator. The medium contained either 10 µg/ml of recombinant type VII collagen or 3 µg/ml of concanavalin A (Sigma) to stimulate the lymphocytes. The plates were washed and the IFN-γ or IL-4 secreted by individual cells were detected in situ by successively reacting each well with biotinylated anti-IFN-γ or anti-IL-4 monoclonal antibody (BD Pharmingen) at 1 µg/ml, following with a 1:1000 dilution of streptavidin-conjugated alkaline phosphatase (Roche). Detection was performed using BCIP/NBT chromogenic substrate (Promega). The reaction was halted by washing with water and spots were counted using a CTL ELISPOT reader. Negative controls were run in parallel using T cells without antigen, and the corresponding scores were subtracted from those of the unknowns.

Anti-C7 LH24 mAb characterization

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LH24 mAb was previously identified to react with epidermal basement membrane [2]. Its specific absence in C7 null RDEB patient skin in our study indicated that it recognized an epitope on C7. To further localize LH24 reactvity on the C7 molecule, we tested LH24 reactivity to enzymatic digests of C7 containing NC1 and NC2 domains by Western blot. NC1 domain containing C7 fragment was produced from digestion of purified C7 with highly purified bacterial collagenase (Worthington) as previously described. [3] NC2 containing C7 fragment was produced following pepsin digestion of purified C7 as previously described.[4]

Electron microscopy:

A 3mm skin punch biopsy was prepared for electron microscopy by immersion in 1.5% glutaraldehyde/1.5% paraformaldehyde in Dulbecco's serum free media (SFM) containing 0.05% tannic acid for a minimum of one hour followed by an extensive rinse in SFM, then post-fixation in 1% OsO4 for 60 minutes. The samples were washed in SFM then dehydrated in a graded series of ethanol to 100%, rinsed in propylene oxide and infiltrated in Spurrs epoxy over a total time of two hours, accelerated via microwave energy. Samples were polymerized at 70°C over 18 hours.

Immuno-electron microscopy:

A 3mm skin punch biopsy sample for immune-electron microscopy were prepared by extensively rinsing in SFM then immersing in mouse IgM LH24 antibody specific to the NC2 region of collagen VII diluted 1:5 in SFM overnight at 4°C, rinsed extensively in SFM, then incubated overnight at 4°C in Goat anti-mouse IgM ultrasmall colloidal gold conjugate (Aurion) diluted 1:3 in SFM. Following an extensive rinse in SFM the samples were exposed to gold enhancement solution (Nanoprobes) 15 minutes on ice, then rapidly warmed to 25°C and incubated an additional 5 minutes. The samples were then rinsed with ice cold SFM, then fixed and embedded as above.

Indirect immuno-fluorescence (IIF):

Human sera was laid upon monkey esophagus and stained with antibodies directed against human IgA, IgM, IgG, and C3. The signal detected at antibody dilutions of 1:40 and higher considered above background.

Direct immuno-fluorescence (DIF):

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Tissue was cut at 5 micrometer and stained with fluorophore-conjugated antibodies to human IgA, IgM, IgG, C3, and fibrinogen. Normal controls were run in parallel.

C7 expression and AF analysis:

A 3mm skin punch biopsy sample was cut at 8 micrometer and analyzed by IIF using anti-type VII collagen polyclonal antibody FNC1 (raised against NC1 domain of C7) or monoclonal antibody LH24 (NC2 domain of C7). Briefly, sections were fixed in a solution of methanol/acetone mixture, permeabilized in detergent and incubated with anti-C7 primary antibody for 1 hour at room temperature (25°C). After extensive washes, secondary antibody conjugated to Alexa Fluor 555 or 488 dye was added and incubated for 1 hour. Cell nuclei labeled with Hoechst 33342 for 10 minutes, washed, and mounted with prolong gold antifade reagent (Life Technologies, Carlsbad, CA). For epidermal markers Keratin 1, Keratin 14 and Loricrin antibodies obtained from Covance (Emeryville, CA). Biopsies were scored positive for C7 expression if continuous linear staining of type VII collagen at the dermal-epidermal junction was detected. Biopsies were scored positive for Anchoring fibrils (AF) if gold conjugate particles were detected representing NC2 domain specific LH24 antibodies at the ultrastructures with characteristic futures of AF including density, thickness, curvature, arching, and looping.

Photography:

For patients 2-4, the Canfield Vectra 3D camera was used to take ~5 images of each graft site from multiple angles. These images were then stitched together to create a comprehensive 3D image. Using Mirror Software (Canfield, Fairfield, NJ) landmarks were selected and numbered to identify corresponding locations on each image, and then melded into a single image. In order to accurately track the graft margins, melded images from follow-up visits were compared to the baseline image, with an overlay of graft outlines from Day 0. Anatomical landmarks (e.g. tattoo dots) were again identified to correctly place the outlines. Additional photographs for patients 2-4 and all images for patient 1 were obtained as needed with digital photography (Canon Powershot).

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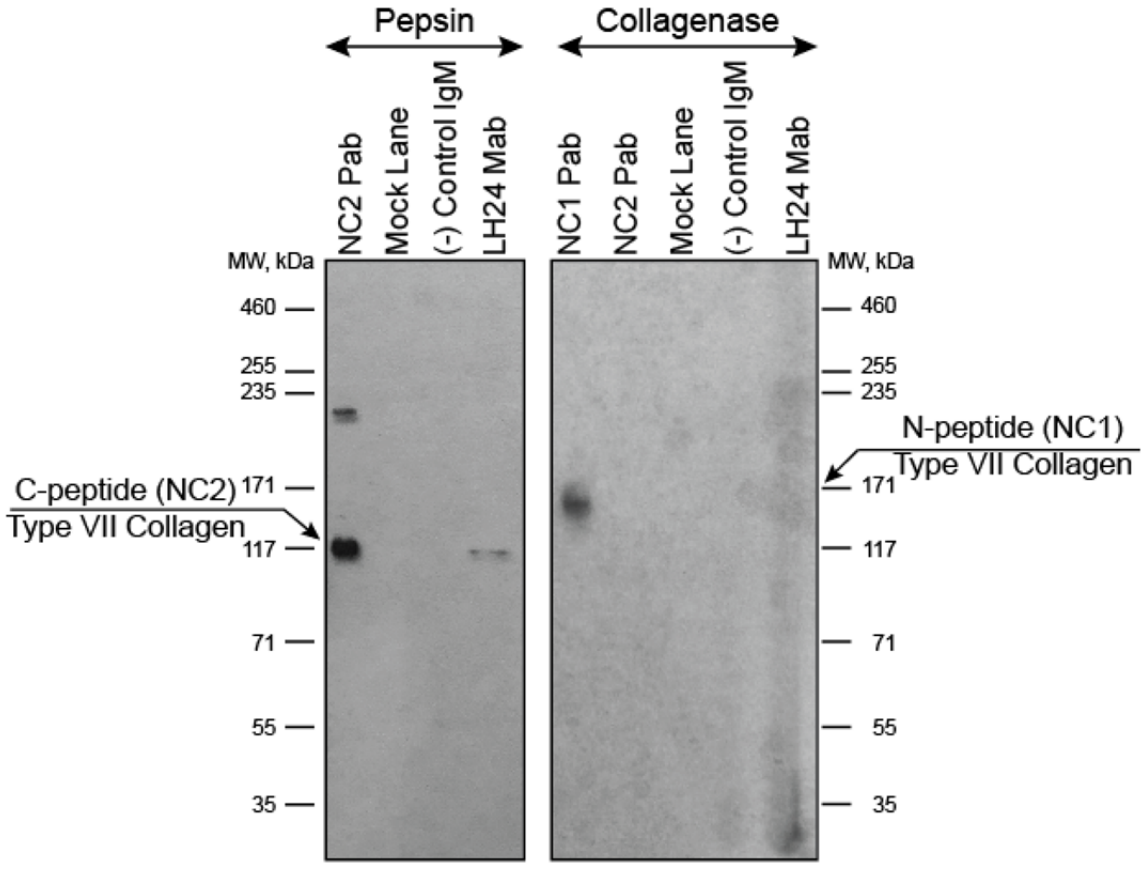
eFigure 1. Anti-type VII collagen LH24 monoclonal antibody characterization

Type VII collagen was produced and purified from a mammalian culture system as previously described [5] and enzymatically digested with pepsin as previously dsecribed[4] to produce a fragment containing the noncollagenous NC2 domain but in which the noncollagenous NC1 domain was absent (Used in panel labeled Pepsin). Purified type VII collagen was alternatively purified with highly purified bacterial collagenase (Worthington) as previously described [3] to produce a type VII collagen fragment containing the noncollagenous NC1 domain but lacking the noncollagenous NC2 domain (used in panel labeled Collagenase). Western blot analysis of these two type VII collagen digestion products reveals LH24 monoclonal antibody (Mab) cross-reactivity to the carboxyl-terminal peptide containing non-collagenous 2 (NC2) domain but not to the collagnase generated fragment in which NC2 is absent. The non-collagenous 2 (NC2) domain presence in the pepsin digested C7 fraction was confirmed with non-collagenous 2 (NC2) specific polyclonal antibody pAb (NC2-10). [6] The NC1 domain in the collagenase digested type VII collagen fraction was identified using NC1 polyclonal antibody pAb.[7] These results confirm that the LH24 antibody recognizes a portion of the type VII collagen containing the noncollagenous NC2 domain, but does not recognize the noncollagenous NC1 domain.



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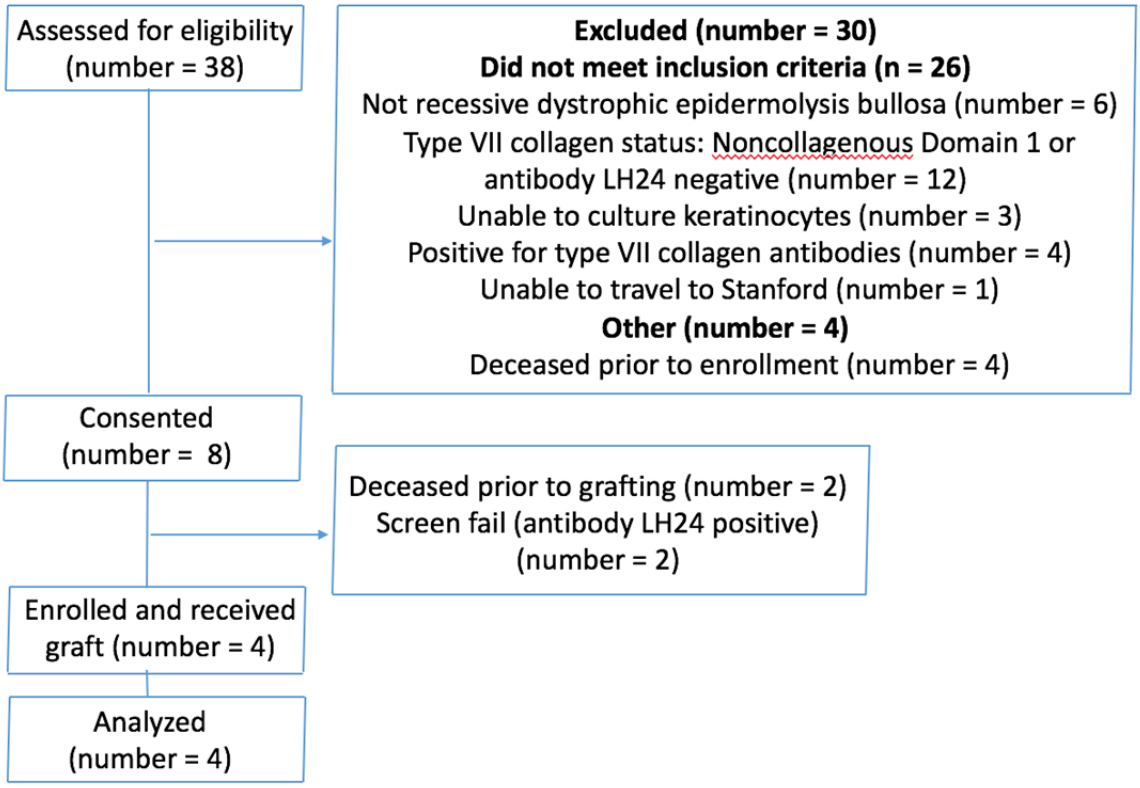
eFigure 2. Patient enrollment diagram



RDEB – recessive dystrophic epidermolysis bullosa, NC1 – non-collagenous domain of type VII collagen, LH24 - Anti-type VII collagen non-collagenous domain two (NC2) monoclonal antibody

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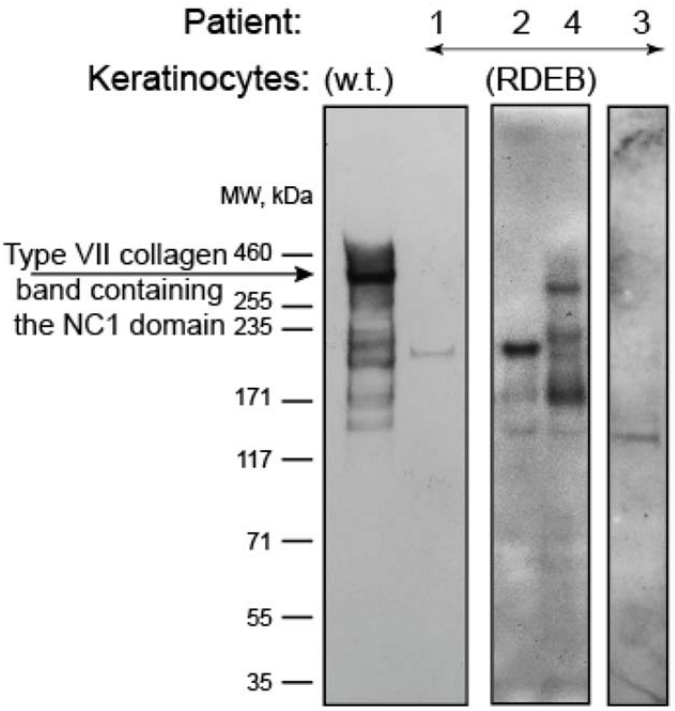
eFigure 3. Baseline type VII collagen expression in recessive dystrophic epidermolysis bullosa (RDEB) patients 1-4

Western blot analyses of cultured primary recessive dystrophic epidermolysis bullosa (RDEB) or normal, wild type (w. t.) keratinocyte supernatant using anti type VII collagen polyclonal antibody specific to non-collagenous 1 (NC1) domain. Note truncated type VII collagen protein expression containing NC1 domain in all patients enrolled in the trial as indicated by distinct reactive bands of lower molecular weight, compared to the band depicted by the arrow in the far left normal wild type (w. t.) lane.



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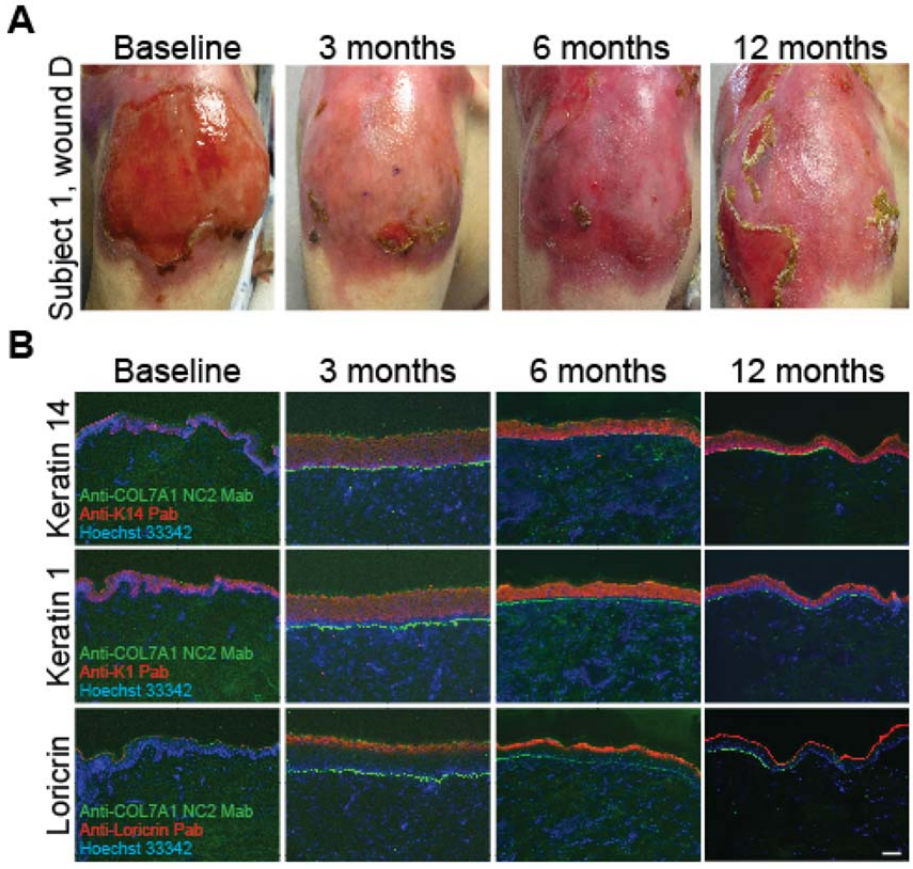
eFigure 4. Patient 1 wounds prior and post grafting

(**A**) Clinical representation of RDEB phenotype before and post graft transplantation. (**B**) IIF analyses of type VII collagen expression in skin grafts. Anti-type VII collagen non-collagenous domain two (NC2) monoclonal antibody (Mab) LH24 (green); Nuclear staining dye Hoechst 33342 (blue); keratin 14 (anti-K14 Pab, orange); keratin 1 (anti-K1 Pab, orange) and loricrin (anti-loricrin Pab, orange). Note linear green staining of type VII collagen at the dermal-epidermal junction of the corrected tissue grafts at all time points. Note correct localization of epidermal markers Keratin 14, keratin 1 and loricrin within corrected tissue grafts. Antibodies raised against indicated proteins labeled as “anti-“. Scale bar, 100 µm



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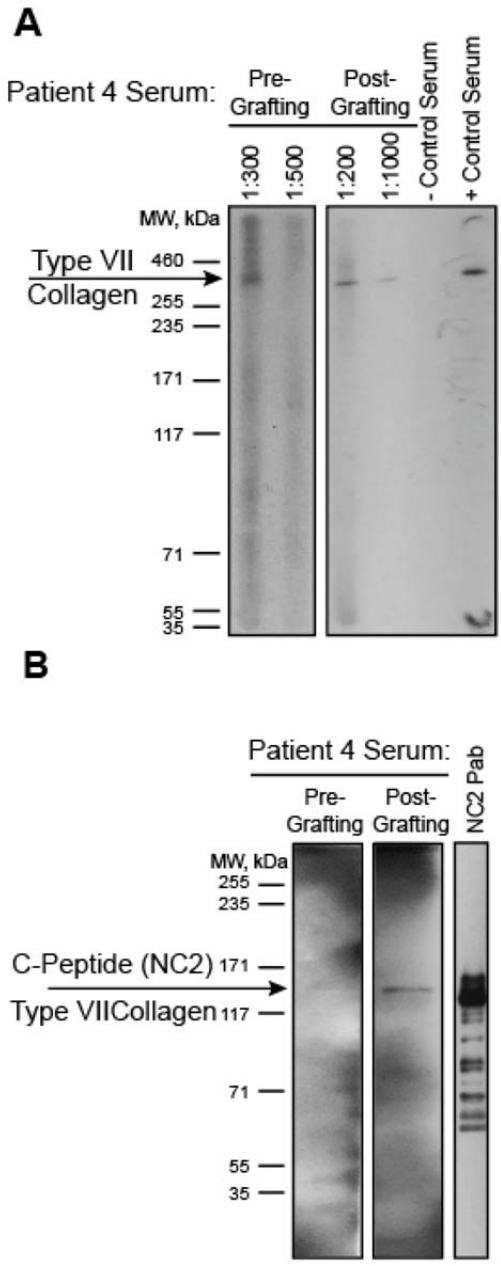
eFigure 5. Patient 4 serum reactivity to type VII collagen

(**A**) Western blot analysis showing cross-reactivity of patient 4 serum to the full-length type VII collagen prior and post graft transplantation (3 months). (**B**) Patient 4 serum obtained pre- and 3 months post grafting is specific to the enzymatically (pepsin) digested type VII collagen protein containing noncollagenous 2 (NC2) domain. Control (right lane) confirming noncollagnenous 2 (NC2) domain presence in the digested type VII collagen fraction using noncollagnenous 2 (NC2) specific polyclonal antibody (Pab) (NC2-10) [6].



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