

Injection of recombinant human type VII collagen restores collagen function in dystrophic epidermolysis bullosa

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Dystrophic epidermolysis bullosa (DEB) is a family of inherited mechano-bullous disorders that are caused by mutations in the type VII collagen gene and for which *ex vivo* gene therapy has been considered. To develop a simpler approach for treating DEB, we evaluated the feasibility of protein-based therapy by intradermally injecting human recombinant type VII collagen into mouse skin and a DEB human skin equivalent transplanted onto mice. The injected collagen localized to the basement membrane zone of both types of tissues, was organized into human anchoring fibril structures and reversed the features of DEB disease in the DEB skin equivalent.

Type VII collagen forms anchoring fibrils, attachment structures in the basement membrane zone (BMZ) of skin that adhere the epidermal layer of skin onto the dermis^{1–3}. Defects in the gene encoding type VII collagen, *COL7A1*, result in DEB^{4,5}, an incurable skin disease characterized by skin fragility, skin blisters, scarring⁶ and a paucity of normal anchoring fibrils¹. DEB can be transmitted in a dominant (DDEB) or recessive manner (RDEB), the recessive forms being more severe. Proposed gene therapy for RDEB has involved *ex vivo* gene delivery in which cultured gene-corrected cells are transplanted onto surgically prepared sites on the skin^{7,8}. This is fraught with technical difficulties and poor graft take. The intradermal injection of gene-corrected RDEB fibroblasts has also been envisioned as a treatment method^{9,10}. To develop a simpler approach, we evaluated the feasibility of protein-based therapy.

We have previously developed a minimal lentiviral transfer vector to express full-length human type VII collagen cDNA and used it to transduce fibroblasts from individuals with RDEB^{7,9}. The recombinant collagen was purified from culture medium of gene-corrected RDEB fibroblasts that contained 2–5 µg/ml of type VII collagen (see Fig. 1a and Supplementary Methods online). The purified recombinant type VII collagen is identical to native type VII collagen¹¹.

We first used an animal model to evaluate the feasibility of protein therapy for RDEB. We injected 20 µg of purified human type VII collagen intradermally into the skin of athymic hairless mice. Every week after injection, we obtained skin biopsies and subjected

them to immunostaining with an antibody specific for human type VII collagen. One week after intradermal injection, the injected collagen was transported from the dermis and incorporated into the mouse's BMZ (Fig. 1b). Mice injected with laminin-1, a control protein, entirely lacked human type VII collagen staining. We confirmed the BMZ localization of the injected type VII collagen by colabeling the same vertical frozen sections of mouse skin with a polyclonal antibody that recognizes both mouse and human type VII collagen (Fig. 1b).

To determine the minimal concentration of protein required for BMZ incorporation, we injected 5, 10, 20 and 40 µg of type VII collagen. We observed a dose-dependent increase in incorporation of the injected protein into the BMZ (Supplementary Fig. 1 online). Further, the injected type VII collagen remained stably incorporated into the BMZ for at least 3 months after a single injection (Fig. 1c). As assessed with optical signal quantification software, there was minimal change (<15%) in the BMZ-localized immunostaining of the injected collagen over the 3-month observation period.

Gene or protein therapy for RDEB may provoke an unwanted immune response against the introduced gene product. To examine if the injected human collagen would induce an immune response and prevent subsequently injected protein from incorporating into the BMZ, we injected recombinant human type VII collagen into immunocompetent SKH mice. Just as in immunodeficient mice, the exogenously injected protein was incorporated into the BMZ and remained stable for at least 6 weeks (Supplementary Fig. 2 online). Sera from ten mice 6 weeks after injection were evaluated for antibodies to type VII collagen by ELISA¹². Although six of the ten mice had such antibodies, none lost weight or showed any untoward effects (data not shown). Notably, the antibodies did not prevent further BMZ incorporation of human type VII collagen injected later into new areas of the mouse's skin (Supplementary Fig. 3 online).

To determine whether the injected type VII collagen could form anchoring fibrils at the BMZ *in vivo*, we carried out immunoelectron microscopy on skin samples from the injected mice using species-specific antibodies that recognize only human type VII collagen. The injected human type VII collagen formed anchoring fibrils—wheat stack-shaped structures 200–600 nm in length—at the BMZ (Fig. 1d). We visualized the anchoring fibrils by labeling them at each end with immunogold particles (by a process previously reported for labeling normal human skin anchoring fibrils¹³), using an antibody that recognizes the N-terminal globular noncollagenous NC1 domain of type VII collagen (fibrils insert into the lamina densa of the BMZ and emanate down into the upper papillary dermis). These data show that injected type VII collagen can form human anchoring fibril structures at the BMZ of the mouse *in vivo*.

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BRIEF COMMUNICATIONS

Dermatologists are skilled at injecting type I collagen into the upper dermis to ameliorate wrinkles. Therefore, it might be feasible to inject human type VII collagen into the dermis of individuals with RDEB to restore their anchoring fibrils and improve their chronic bullous disease. To evaluate this approach, we injected recombinant type VII collagen intradermally into RDEB skin tissues regenerated on immunodeficient mice as previously described¹⁴. This system generates human skin tissues that retain the RDEB disease phenotype¹⁵. Regenerated RDEB skin not injected with protein showed histological evidence of dermal-epidermal separation and entirely lacked human type VII collagen staining at the BMZ, both characteristics of RDEB skin (Fig. 2a,b). However, the intradermal injection of recombinant type VII collagen into RDEB skin corrected the subepidermal blistering and restored type VII collagen expression at the

BMZ, in a pattern similar to that in skin regenerated from normal control cells, for at least 2 months after a single injection (Fig. 2a–c). Immunoelectron microscopy using species-specific antibodies to human type VII collagen showed that the injected type VII collagen also restored anchoring fibrils in the RDEB skin (Fig. 2d). Taken together, these data indicate that protein-based therapy by intradermal injection of type VII collagen can stably correct *in vivo* the abnormal dermal-epidermal separation of RDEB and restore type VII collagen and anchoring fibrils at the BMZ.

In summary, our studies show that intradermally injected recombinant human type VII collagen is transported from the dermis and incorporates into the BMZ, where it forms human anchoring fibril structures. We did not observe the injected human type VII collagen distributed diffusely throughout the dermis of either mouse skin or

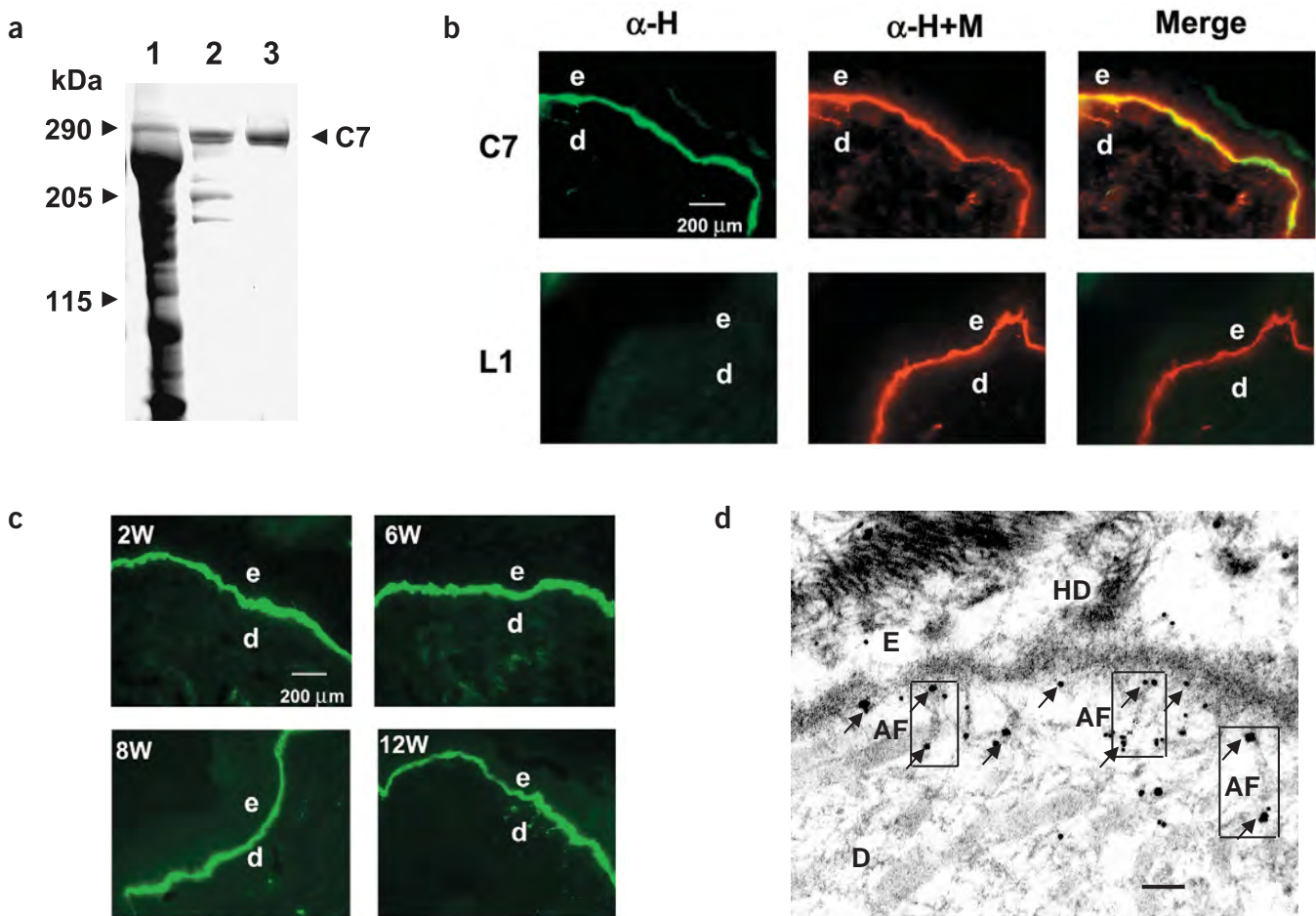
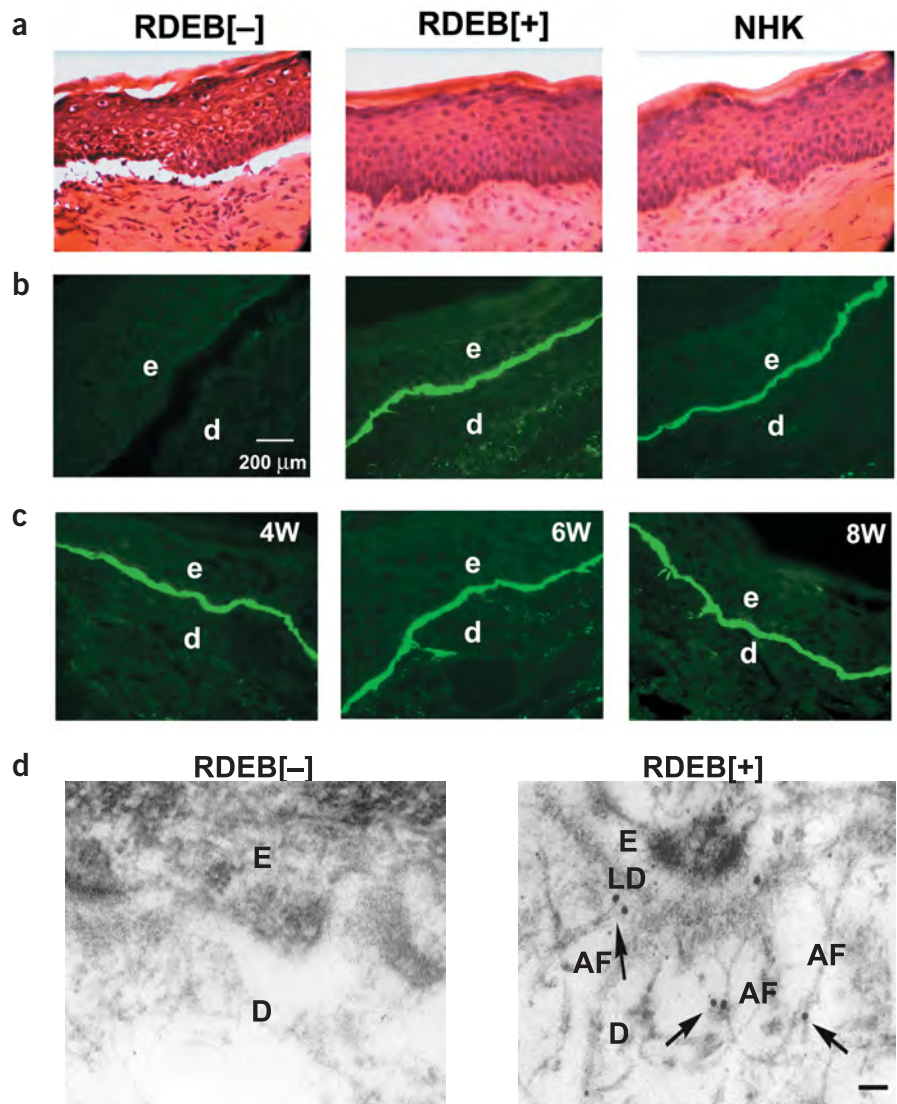


Figure 1 Intradermally injected human type VII collagen stably incorporates into the mouse's BMZ. (a) Purification of recombinant type VII collagen from gene-corrected RDEB cells. 6% SDS-PAGE and Coomassie Blue staining. Lane 1, ammonium sulfate precipitated conditioned media from gene-corrected RDEB fibroblasts; lanes 2 and 3, recombinant type VII collagen solubilized by 150 mM NaCl and type VII collagen purified using Q-Sepharose chromatography, respectively. The positions of the molecular weight markers and 290-kDa type VII collagen are indicated. See **Supplementary Methods** online for details of purification of type VII collagen. (b) Immunofluorescence staining of hairless immunodeficient mouse skin 1 week after intradermal injection of 20 µg of purified recombinant type VII collagen (C7) or control protein (laminin-1, L1). The tissue was labeled with either a monoclonal antibody specific for human type VII collagen (α-H, green) or a rabbit polyclonal antibody that recognizes both mouse and human type VII collagen (α-H+M, red). (c) Sustained incorporation of the injected human type VII collagen at the mouse's BMZ. Skin biopsies from athymic hairless mice intradermally injected with 20 µg of recombinant type VII collagen were taken at the times indicated and stained with a monoclonal antibody specific for human type VII collagen. e, epidermis; d, dermis; W, weeks. (d) Immunoelectron microscopy of mouse skin injected with recombinant human type VII collagen. Immunogold labeling of mouse skin after injection with human type VII collagen was performed using a human-specific anti-type VII collagen antibody. Arrows denote gold particle-labeled NC1 domains of injected human type VII collagen. The boxes highlight representative anchoring fibrils labeled with gold particles at both NC1 ends. AF, anchoring fibrils; D, dermis; E, epidermis; HD, hemidesmosome. Scale bar, 100 nm.

Figure 2 Intradermally injected human type VII collagen incorporates into the BMZ of regenerated human RDEB skin tissue *in vivo*. (a) Histological appearance of human skin tissues regenerated on athymic hairless mice from either normal human keratinocytes (NHK) or keratinocytes of an individual with RDEB, 2 weeks after injection with PBS (RDEB[−]) or 20 μg of recombinant type VII collagen (RDEB[+]). (b) Immunofluorescence staining using a monoclonal antibody specific for human type VII collagen of regenerated normal control skin (right), RDEB skin (left) or RDEB skin 2 weeks after an intradermal injection of 20 μg purified recombinant type VII collagen (middle). (c) Biopsies from regenerated RDEB skin injected intradermally with 20 μg of recombinant type VII collagen were taken at the times indicated (W, weeks) and stained with a monoclonal antibody specific for human type VII collagen. e, epidermis; d, dermis. (d) Immunogold labeling of RDEB skin or RDEB skin injected with human type VII collagen was done using a human-specific anti-type VII collagen antibody. Arrows, gold particle-labeled NC1 domains of injected human type VII (anchoring fibril) collagen; AF, anchoring fibrils; D, dermis; E, epidermis; LD, lamina densa. Scale bar, 100 nm.



regenerated RDEB skin. Rather, it was localized to the BMZ between the dermis and epidermis. It is possible that the injected type VII collagen reaches the epidermal-dermal junction by diffusion and then selectively adheres to the BMZ because of its great affinity for laminin-5 and type IV collagen within the BMZ¹¹. Defining the potential advantages and drawbacks of protein-based therapy as compared with conventional gene therapy approaches is of considerable interest for future studies. Much as bovine type I collagen (Zyderm) is injected into humans to reduce wrinkles, one could perhaps inject human type VII collagen into the skin of individuals with DEB. This methodology may also provide a useful therapeutic strategy for other skin disorders caused by defects in genes encoding other structural proteins in the skin.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

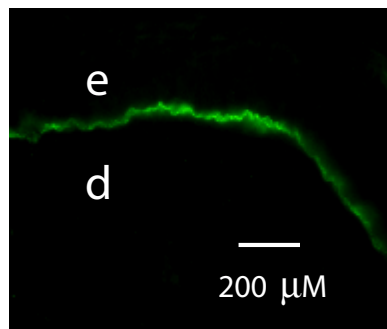
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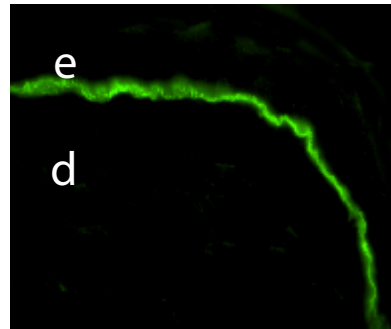
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Supplementary Figure 1

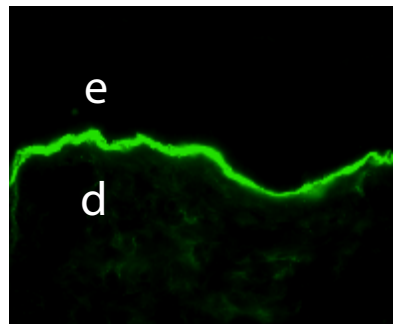
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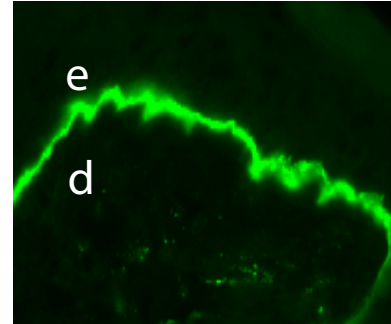
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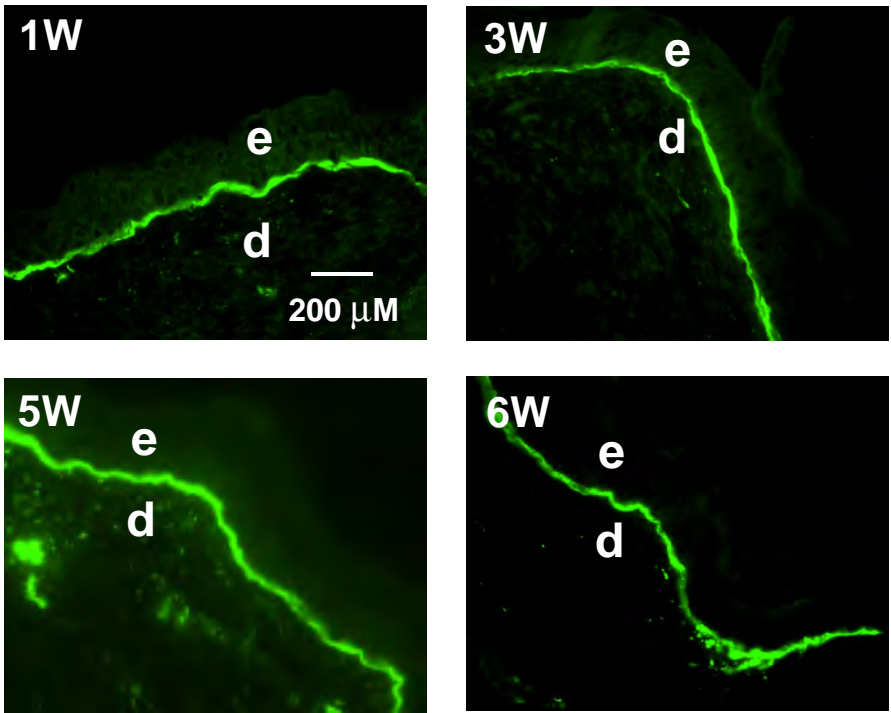


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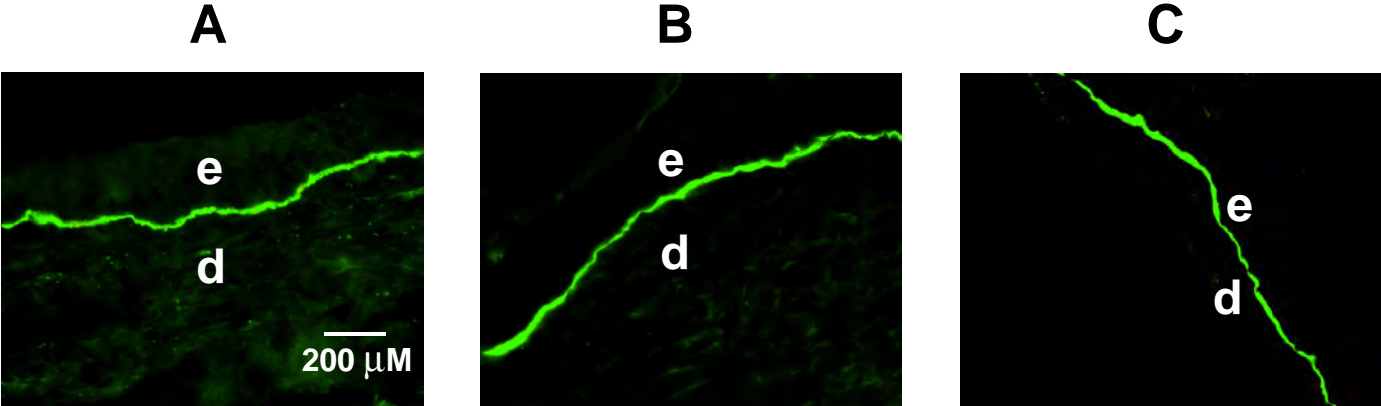
Supplementary Figure 1 Dose-dependent deposition of injected human type VII collagen at the mouse's BMZ. Immunofluorescence staining with an antibody specific for human type VII collagen was performed on mouse skin one week after being injected with various doses of recombinant type VII collagen as indicated. Note a dose-dependent increase in the incorporation of human type VII collagen at the mouse BMZ.

Supplementary Figure 2



Supplementary Figure 2 Sustained incorporation of human type VII collagen at the BMZ of hairless immunocompetent mice after a single intradermal injection. Skin biopsies from hairless immunocompetent mice intradermally injected with 20 μ g of recombinant type VII collagen were taken at the times indicated and stained with a monoclonal antibody specific for human type VII collagen. e, epidermis; d, dermis.

Supplementary Figure 3



Supplementary Figure 3 Incorporation of subsequently injected type VII collagen into the mouse's BMZ regardless the presence of anti-type VII collagen antibodies. We selected three immunocompetent mice (A-C) who had been injected with human type VII collagen and were found by ELISA to have anti-human type VII collagen antibodies in their blood. We then re-injected these antibody-positive mice with 20 µg of purified recombinant human type VII collagen at a completely different skin site.

Immunofluorescence staining with an antibody specific for human type VII collagen was performed on mouse skin one week after injection. Interestingly, we found that the newly injected human type VII collagen protein was still transported from the dermis and incorporated into the mouse's BMZ. These data indicate that the presence of anti-type VII collagen antibodies do not prevent additional BMZ incorporation of subsequently injected type VII collagen.

Supplementary Methods

Protein purification. Recombinant type VII collagen was purified from serum free media of RDEB fibroblasts stably transduced with a lentiviral vector coding for full-length type VII collagen as described¹. Briefly, serum-free media were equilibrated to 5 mM EDTA, 50 μ M PMSF and 50 μ M NEM and precipitated with 300 mg/ml ammonium sulfate at 4⁰ C overnight with stirring². Precipitated proteins were collected by centrifuging at 1.2 X 10⁶ g/min for 1 hr, resuspended and dialyzed in Buffer A (65 mM NaCl, 25 mM Tris-HCl, pH 7.8). Following dialysis, insoluble material was collected by centrifugation at 8,600 g for 20 min, and the pellet redissolved in Buffer B (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM NEM, 2 mM PMSF). The solution was clarified as above, and the supernatant, S1', was passed over a Q-sepharose column (Pharmacia, Inc., Piscataway, NJ) equilibrated in the same buffer. Elution was then carried out with a linear gradient from 0.2 to 1.0 M NaCl of appropriate volume size. The type VII collagen was eluted at 0.7 - 1 M NaCl.

Intradermal injection of recombinant type VII collagen into mice. We intradermally injected 5-40 μ g of purified recombinant type VII collagen suspended in 100 μ l of phosphate buffered saline into the dorsal back skin of 4-6 week old athymic hairless mice (The Jackson Laboratory, Bar Harbor, ME) or hairless SKH1 immunocompetent mice (the Charles River Laboratories), using a thirty gauge needle. We used four or more animals for each experimental

group. One to 12 weeks after injection, mouse skin biopsies were obtained and subjected to immunostaining using an antibody specific for human type VII collagen (clone LH 7.2; Sigma, St. Louis, MO) or a rabbit polyclonal antibody recognizing both human and mouse type VII collagen as described below. All animal studies were conducted using protocols approved by the University of Southern California Institutional Animal Use Committee.

Intradermal injection of recombinant type VII collagen into regenerated RDEB skin tissue on mice. For human skin studies, skin of RDEB patients and normal controls was generated using either early-passage RDEB keratinocytes or normal human keratinocytes and grafted onto immuno-deficient mice as previously described⁴. Devitalized porcine dermal substrate was used as described to avoid immune cross-reactivity of the antibodies to type VII collagen used in these studies. At least 6 grafts were regenerated for each of the two RDEB patients studied. These grafts were injected with either PBS alone (three grafts) or PBS containing type VII collagen (three grafts). Three normal control human skin grafts were also regenerated from the keratinocytes of non-diseased human subjects. Four weeks after grafting, 20 µg of purified recombinant type VII collagen resuspended in 100 µl PBS was injected intradermally into the center of each RDEB graft using a thirty-gauge needle. Two to 8 weeks after injection, biopsies and immunofluorescence staining were performed on the regenerated human skin tissues using an antibody specific for human type VII collagen (clone NP185; Chemicon Inc., Temecula, CA) as described below. All animal studies were conducted using protocols approved by the University of Southern California Institutional Animal Use Committee.

Immunofluorescence staining and ultrastructural analysis of tissue. Five-micrometer thick sections of the OCT-embedded tissues were cut on a cryostat, fixed for 5 min in cold acetone, and air-dried. Sections from mice skin intradermally injected with recombinant type VII collagen were incubated with a monoclonal antibody against human type VII collagen (clone LH 7.2; Sigma, St. Louis, MO) or a rabbit polyclonal antibody that recognizes both mouse and human type VII collagen, followed by a FITC-conjugated goat anti-mouse Ig G or Cy3-conjugated goat anti-rabbit Ig G (Sigma, St. Louis, MO). Working dilutions were 1:100 for the primary antibody and 1:200 for the secondary antibody. Immunolabeling of the tissue was performed using standard immunofluorescence methods as described previously⁵. To assess human anchoring fibril formation and ultrastructure, 50 micro-meter sections were fixed in 0.1% glutaraldehyde, rinsed in 0.15 M Tris, then incubated with an antibody specific for human type VII collagen (NP185, gift of Dr. Lynn Sakai, Shriners Hospital for Children, Portland, Oregon), followed by a 1 nm gold secondary antibody and enhancement as described⁶.

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