Molecular Pathogenesis of Genetic and Inherited Diseases

Keratinocyte-/Fibroblast-Targeted Rescue of *Col7a1*-Disrupted Mice and Generation of an Exact Dystrophic Epidermolysis Bullosa Model Using a Human COL7A1 Mutation

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Recessive dystrophic epidermolysis bullosa (RDEB) is a severe hereditary bullous disease caused by mutations in COL7A1, which encodes type VII collagen (COL7). Col7a1 knockout mice (COL7^{m-/-}) exhibit a severe RDEB phenotype and die within a few days after birth. Toward developing novel approaches for treating patients with RDEB, we attempted to rescue COL7^{m-/-} mice by introducing human COL7A1 cDNA. We first generated transgenic mice that express human COL7A1 cDNA specifically in either epidermal keratinocytes or dermal fibroblasts. We then performed transgenic rescue experiments by crossing these transgenic mice with COL7^{m+/-} heterozygous mice. Surprisingly, human COL7 expressed by keratinocytes or by fibroblasts was able to rescue all of the abnormal phenotypic manifestations of the COL7^{m-/-} mice, indicating that fibroblasts as well as keratinocytes are potential targets for RDEB gene therapy. Furthermore, we generated transgenic mice with a premature termination codon expressing truncated COL7 protein and performed the same rescue experiments. Notably, the COL7^{m-/-} mice rescued with the human COL7A1 allele were able to survive despite demonstrating clinical manifestations very

similar to those of human RDEB, indicating that we were able to generate surviving animal models of RDEB with a mutated human COL7A1 gene. This model has great potential for future research into the pathomechanisms of dystrophic epidermolysis bullosa and the development of gene therapies for patients with dystrophic epidermolysis bullosa. (Am J Pathol 2009, 175:2508–2517; DOI: 10.2353/ajpath.2009.090347)

Dystrophic epidermolysis bullosa (DEB) is clinically characterized by mucocutaneous blistering in response to minor trauma, followed by scarring and nail dystrophy. The blistering occurs along the epidermal basement membrane zone (BMZ) just beneath the lamina densa at the level of the anchoring fibrils. The inheritance of DEB can be autosomal dominant (DDEB) or autosomal recessive (RDEB), each comprising subtypes of different clinical presentations and severities.¹ Both DDEB and RDEB are known to be caused by mutations in the COL7A1 gene encoding type VII collagen (COL7), the major component of anchoring fibrils.² The most severe RDEB subtype, the Hallopeau-Siemens subtype, shows a complete lack of expression of type VII collagen, whereas a less severe RDEB subtype, the non-Hallopeau-Siemens subtype, shows some collagen expression. The clinical fea-

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tures of DDEB are, in general, milder than those of RDEB and tend to improve with age. The molecular mechanisms of DEB have been thoroughly investigated, and precise diagnosis and estimation of prognosis is now possible. There is no specific treatment for different forms of DEB, and the current focus of research is to develop more effective treatments for this group of blistering disorders.

Corrective gene therapy whereby normal COL7 is introduced into the patients' cells, has great potential as a treatment for DEB. However, several obstacles must be overcome before its clinical therapeutic application. First, there have been no useful DEB animal models that reproduce the human mutated gene for experiments. Although COL7 knockout mice have been generated, most of such mice die within a few days of birth, and none survive more than 2 weeks.³ A surviving DEB mouse that was reported recently was the DEB hypomorphic mouse model.⁴ These mice, which had about 10% of the normal mouse COL7, did not show the abnormal form and function of anchoring fibrils seen in human patients of RDEB. Second, no studies have examined in detail whether the introduction of the human COL7 gene into DEB mouse cells can rescue the DEB phenotype without causing adverse effects in a living DEB model. Third, there is controversy over which cells may serve as optimal targets in gene therapies for DEB. Several studies have targeted keratinocytes, because the cells that secrete COL7 are mainly keratinocytes and to a lesser extent fibroblasts.^{5,6} However, we and others have recently reported that injection of gene-transferred fibroblasts into the skin can efficiently restore COL7 expression in the dermal-epidermal junction *in vitro*.^{6–8} Furthermore, intra-dermal injection of allogeneic fibroblasts into skin of patients with RDEB skin was shown to result in enhanced COL7 expression in selected patients.⁹ Therefore, we need to compare keratinocytes and fibroblasts to clarify their efficacy as target cells in an *in vivo* model system of RDEB.

To address these issues, we generated transgenic mice with human COL7A1 under different promoters and performed transgenic rescue experiments on the Col7a1^{tm-/-} background using those transgenic mice. Furthermore, to develop a DEB model that accurately reproduces human DEB not only in terms of clinical manifestations but also in terms of gene mutation, we also introduced a mutated human COL7A1 gene into this mouse model system and created human mutant gene-expressing rescued mice corresponding to the surviving animal of DEB. Our results advance our understanding of the function and biology of COL7.

Materials and Methods

Generation of Transgenic Mice

Human full-length COL7A1 cDNA was constructed from several overlapping cDNA clones (Sawamura et al, 2002). We used a pCMV β expression vector (Invitrogen, Carlsbad, CA) that contained the cytomegalovirus (CMV)

Figure 1. Epidermis- or dermis-specific expression of the human COL7A1 full-length cDNA in the transgenic mice. **A:** Three expression vectors for transgenic mice were constructed using the promoters of human K14, mouse col1a2, and CMV. The vector contains the SV40 splice donor/splice acceptor (SD/SA) site and the SV40 polyadenylation (polyA) signal. **B:** We obtained epidermis and dermis from the K14Tg mice (COL7^{K14-h+}), col1a2Tg mice (COL7^{col1-h+}), and CMVTg mice (COL7^{CMV-h+}) and then examined human COL7A1 mRNA expression by RT-PCR analysis. Molecular weight markers (M) are a 100-bp DNA ladder. **C:** Expression of COL7 was also investigated by Western blot analysis using anti-human monoclonal antibody LH7.2. Molecular weight markers are a biotinylated protein ladder.

promoter, the simian virus 40 (SV40) splice donor/splice acceptor site, the *lacZ* gene, and the SV40 polyadenylation signal. We selected human keratin 14 (K14),¹⁰ the mouse pro- α 2 chain of type I collagen (col1a2),¹¹ or the CMV promoter for epidermis-specific, dermis-specific, or ubiquitous expression of the transgene, respectively. We first modified pCMV β by replacing LacZ with human full-length COL7A1 cDNA, and the CMV promoter with the human K14 or the mouse col1a2 gene. Finally, we produced three COL7A1 constructs for transgenic mice (Figure 1A). They were digested with appropriate restriction enzymes, purified, and introduced into BDF1 oocytes, which were subsequently transplanted into the recipient mice. Founders were bred to wild-type C57BL/6 females. To confirm germline transmission, PCR analyses on genomic DNA were performed (forward, 5'-CTCAGTG-GATGTTGCCCTT-3'; reverse, 5'-TAAGAACACAATGT-CAGCGG-3') using specific primers and the following thermal cycling parameters: 94°C for 5 minutes, 94°C for 45 seconds, and 56°C for 45 seconds; followed by 35 cycles at 72°C for 45 seconds and 72°C for 7 minutes. The transgenic (Tg) mice with K14, col1a2, and CMV

Table 1. Summary of the Genetically Engineered Mice Involved in This Study

Mouse	Genotype	Phenotype
COL7 ^{m-/-}	Knockout mouse with targeted disruption of the mouse <i>Col7a1</i> encoding mouse type VII collagen	RDEB (severe disease phenotype)
COL7 ^{m+/-}	<i>Col7a1</i> heterozygous knockout mouse	Clinically normal
COL7 ^{K14-h+}	Transgenic mouse with human <i>COL7A1</i> driven by the human K14 promoter	Clinically normal
COL7 ^{col1-h+}	Transgenic mouse with human <i>COL7A1</i> driven by the promoter of the gene encoding mouse pro- α 2 chain of type I collagen	Clinically normal
COL7 ^{CMV-h+}	Transgenic mouse with human <i>COL7A1</i> driven by the ubiquitous CMV promoter	Clinically normal
COL7 ^{m-/-, K14-h+}	<i>Col7a1</i> knockout mouse rescued by human <i>COL7A1</i> with the human K14 promoter	Clinically normal
COL7 ^{m-/-, col1-h+}	<i>Col7a1</i> knockout mouse rescued by human <i>COL7A1</i> with the mouse pro- α 2 chain of type I collagen promoter	Clinically normal
COL7 ^{m-/-, CMV-h+}	<i>Col7a1</i> knockout mouse rescued by human <i>COL7A1</i> with the CMV promoter	Clinically normal
COL7 ^{K14-Δh+}	Transgenic mouse with mutated human type VII collagen with 7528delG mutation under the human K14 promoter	Clinically normal
COL7 ^{m-/-, K14-Δh+}	<i>Col7a1</i> knockout mouse with the mutated human type VII collagen with 7528delG	RDEB (moderate disease phenotype)

promoters were designated as K14Tg mice (COL7^{K14-h+}), col1a2Tg mice (COL7^{col1-h+}), and CMVTg mice (COL7^{CMV-h+}), respectively (Table 1).

Transgenic Rescue Experiment

Transgenic mice with different promoters (COL7^{K14-h+}, COL7^{col1-h+}, and COL7^{CMV-h+}) were crossbred to heterozygous *col7a1* knockout mouse (COL7^{m+/-}) generated by Heinonen et al³ to create heterozygous mice carrying human *COL7A1* cDNA. Then these mice were mated again with COL7^{m+/-} mice to obtain a mouse that harbored the human COL7 gene in a *col7a1* knockout background. The resulting transgenic rescue mice, each with either the K14, col1a2, or CMV promoter, were, respectively, designated as COL7^{m-/-, K14-h+}, COL7^{m-/-, col1-h+}, and COL7^{m-/-, CMV-h+} (Table 1). The rescued mice (COL7^{m-/-, K14-h+}, COL7^{m-/-, col1-h+}, and COL7^{m-/-, CMV-h+}) were analyzed by histopathological, immunofluorescence, and immunoblot analyses as described below. Whole-skin samples from the rescued mice were used for the immunoblot analysis.

RT-PCR and Western Blot Analysis

Mouse skin was obtained from the back of each mouse and incubated with 10 mg/ml dispase for 8 hours at 4°C to separate the epidermis and dermis. The epidermal and dermal sheets were minced, and total RNA was extracted using an RNeasy RNA extraction kit (Qiagen, Hilden, Germany). The cDNA was synthesized with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Grand Island, NY) and subjected to PCR, using specific primers (forward, 5'-CTCAGTGGATGTTGCCTTTA-3'; reverse, 5'-TAAGAACAACAATGTCTAGCGG-3') and the following thermal cycling parameters: 94°C for 5 minutes, 94°C for 1 minute, and 56°C for 1 minute; followed by 35 cycles at 72°C for 1 minute and 72°C for 7 minutes.

For Western blot analysis, the epidermal and dermal sheets were mixed with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), homogenized, and centrifuged at

15,000 $\times$ g. The supernatant of each sample was separated on a 5% polyacrylamide gel under reducing conditions. Immunoblotting analysis was performed by incubation with the LH7.2 monoclonal antibody (1:1000) for 18 hours at 4°C and then with secondary goat anti-mouse IgG antibodies conjugated with peroxidase (1:2000) for 1 hour at 37°C. The resultant complexes were processed using the Phototope HRP Western Blot Detection System (Cell Signaling Technology, Beverly, MA) according to the manufacturer's protocol.

Histopathological, Immunofluorescence, Ultrastructural, and Immunoelectron Microscopic Analyses

Mouse skin samples were fixed in 10% formalin neutral buffer solution for paraffin embedding or were immediately frozen in OCT compound and stored at -80°C. Paraffin-embedded sections were cut to 5 μ m and stained with H&E solution. Alternatively, the LH7.2 monoclonal antibody against the NC-1 amino-terminal domain of COL7 (Chemicon, Temecula, CA) was used for immunofluorescence staining on frozen sections from tissue samples embedded in OCT compound. The bound antibodies were detected with fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Nuclear counterstaining with propidium iodide was performed in some immunofluorescence labeling experiments.

For electron microscopic examination, skin specimens were fixed in 5% glutaraldehyde, postfixed in 1% osmium tetroxide, and stained en block in uranyl acetate. They were dehydrated in a graded ethanol series and embedded in Araldite 6005. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. The sections were examined with a transmission electron microscope (H-7100; Hitachi, Tokyo, Japan) at 75 kV. For semiquantitative morphometric analysis, the number of anchoring fibrils on electron micrographs was counted and the number of anchoring fibrils per unit length of lamina

densa was estimated as number of anchoring fibrils/ $1\ \mu\text{m}$ of lamina densa. Minimal anchoring fibril features required for quantification were the presence of an arch structure of fibrils inserted into the dermis from the lamina densa. Twenty electron microscopic sections were examined for each mouse line. For immunoelectron microscopic analysis, skin samples were cryofixed with liquid propane cooled with nitrogen, cryosubstituted at -80°C , and low temperature-embedded at -60°C in Lowicryl K11M resin before undergoing UV polymerization. Ultra-thin sections were cut to 90 nm thickness. The LH7.2 monoclonal antibody was used as the primary antibody, and then a goat anti-rabbit IgG 10-nm gold-conjugated secondary antibody was used (Amersham, Poole, UK). The sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope.

Transgenic Rescue Experiment with the Human Mutated Gene

We generated a full-length human *COL7A1* cDNA containing the c.7528delG mutation and replaced the normal human *COL7A1* cDNA K14 promoter construct (Figure 1A) with the human mutated *COL7A1* cDNA (c.7528delG). The guanine at 7528 is at the end of the collagenous domain of COL7, and the mutation creates a premature stop codon at 18 bp downstream. The expressed protein is a truncated COL7 lacking the NC-2 domain at the C terminus. Using the same techniques as described above, we produced transgenic mice by microinjection, screening PCR, and germline transmission. The transgenic mice ($\text{COL7}^{\text{K14-h+}}$) were crossbred to heterozygous *col7a1* knockout mouse ($\text{COL7}^{\text{m-/-}}$). Then these mice ($\text{COL7}^{\text{m+/-, K14-}\Delta\text{h+}}$) were intercrossed to obtain a mouse that harbored the mutated human COL7 gene in a *col7a1* knockout background (Table 1). The rescued mice ($\text{COL7}^{\text{m-/-, K14-}\Delta\text{h+}}$) were analyzed by histopathological, immunofluorescence, and immunoblot analyses as described above.

Results

Generation of Transgenic Mice Showing Keratinocyte- or Fibroblast-Targeted Expression of Human COL7

To allow selective expression of human *COL7A1* in epidermal keratinocytes or in dermal fibroblasts, we used human K14 and mouse *col1a2* promoters, which have been shown to specify epidermal and dermal expression in mice, respectively.^{10,11} The mice with K14 or *col1a2* promoters were designated as K14Tg mice ($\text{COL7}^{\text{K14-h+}}$) and *col1a2*Tg mice ($\text{COL7}^{\text{col1-h+}}$), respectively (Figure 1A). In addition, the ubiquitous CMV promoter was used to generate transgenic mice with both epidermal and dermal expression (CMVTg mice: $\text{COL7}^{\text{CMV-h+}}$) (Figure 1A). To demonstrate tissue-specific expression, we obtained epidermis and dermis from the mice and deter-

Figure 2. Both epidermis- and dermis-targeted transgene products of human COL7 molecules were precisely localized in the dermoepidermal junction in the transgenic mice. Immunofluorescence staining with the anti-human COL7 monoclonal antibody LH7.2 showed no human COL7 immunolabeling in the dermoepidermal junction of the wild-type mouse skin (A and B). Skin samples from all three transgenic mouse lines (keratinocyte-targeted K14Tg mice ($\text{COL7}^{\text{K14-h+}}$) (C and D), fibroblast-targeted *col1a2*Tg mice ($\text{COL7}^{\text{col1-h+}}$) (E and F), and CMVTg mice with ubiquitous COL7 expression ($\text{COL7}^{\text{CMV-h+}}$) (G and H) showed human COL7 linear staining at the epidermal BMZ (white arrowheads). $\text{COL7}^{\text{K14-h+}}$ mouse skin revealed additional punctate staining in epidermal keratinocytes, and $\text{COL7}^{\text{col1-h+}}$ mouse skin showed additional diffuse staining in dermal fibroblasts. Dotted lines demarcate the skin surface. Left column (A, C, E, and G), low-power view; right column (B, D, F, and H), high-power view. Human COL7 immunolabeling, green (fluorescein isothiocyanate); nuclear stain, red (propidium iodide). Scale bars = 50 μm .

mined *COL7A1* mRNA expression by RT-PCR analysis using primers specific for human transcripts. The results show that $\text{COL7}^{\text{K14-h+}}$ mice *COL7A1* mRNA expression is restricted to the epidermis and mRNA expression of *COL7A1* in $\text{COL7}^{\text{col1-h+}}$ mice is restricted to the dermis. Expression of epidermal and dermal *COL7A1* mRNA was detected in $\text{COL7}^{\text{CMV-h+}}$ mice (Figure 1B). Western blot analysis also shows epidermal or dermal expression mostly consistent with the specific promoters, except for a weak COL7 band detected in the dermal component from $\text{COL7}^{\text{K14-h+}}$ mice (Figure 1C). A small amount of COL7 secreted by epidermal keratinocytes moves into the dermal side. The weak COL7 band in the dermal component from $\text{COL7}^{\text{K14-h+}}$ mice probably reflects the translocated COL7 peptides.

Immunofluorescence study using LH7.2 anti-human COL7 monoclonal antibody showed the linear epidermal

BMZ staining in all three transgenic mice lines (Figure 2, A and B, wild-type; Figure 2, C and D, COL7^{K14-h+}; Figure 2, E and F, COL7^{col1-h+}; and Figure 2, G and H, COL7^{CMV-h+}). COL7^{K14-h+} revealed additional punctate staining in epidermal keratinocytes (Figure 2, C and D), and COL7^{col1-h+} revealed additional diffuse staining in dermal fibroblasts (Figure 2, E and F). In the course of the transgenic mouse experiments, we obtained several lines of mice and were able to generate offspring in COL7^{K14-h+}, COL7^{col1-h+}, and COL7^{CMV-h+} lineages. In each transgenic line, we selected the mouse with the most robust COL7A1 expression for the subsequent rescue experiments.

Keratinocyte-/Fibroblast-Targeted Transgenic Rescue of COL7 Knockout Mice

Col7a1 knockout mice (COL7^{m-/-}) exhibit a severe, recessive DEB phenotype, and these mice die within a few days after birth. We initiated transgenic rescue experiments of COL7^{m-/-} mice by mating COL7^{m+/-}, COL7^{col1-h+}, or COL7^{CMV-h+} transgenic mice. After further crossing, transgenic mice on a col7a1 knockout background (COL7^{m-/-}, K14-h+, COL7^{m-/-}, col1-h+, and COL7^{m-/-}, CMV-h+) were generated, and they showed expression of human COL7 under the different promoters. All three different rescued mice (COL7^{m-/-}, K14-h+,

Figure 3. Keratinocyte-/fibroblast-targeted human COL7A1 transgene can rescue COL7 knockout mice. A wild-type mouse showed a normal phenotype (**A**) and intact dermoepidermal junction (**B**) without human COL7 expression (**C**, white arrowheads). A COL7^{m-/-} mouse had a severe DEB phenotype (**D**) and apparent dermoepidermal separation (**E**, asterisks) without human COL7 (**F**, white arrowheads). All three rescued mice (keratinocyte-targeted rescued COL7^{m-/-}, K14-h+ [**G-I**], fibroblast-targeted rescued COL7^{m-/-}, col1-h+ [**J-L**], and ubiquitous CMV promoter-driven rescued COL7^{m-/-}, CMV-h+ [**M-O**]) showed no DEB phenotype (**G**, **J**, and **M**) and an intact dermoepidermal junction (**H**, **K**, and **N**). Immunofluorescence labeling revealed human COL7 in the basement membrane zone (white arrowheads) in skin sections from all three rescued mice (**I**, **L**, and **O**). Skin from the keratinocyte-targeted rescued COL7^{m-/-}, K14-h+ mouse showed additional punctate staining in epidermal keratinocytes (**I**), and skin from the fibroblast-targeted rescued COL7^{m-/-}, col1-h+ mouse revealed additional diffuse staining in dermal fibroblasts (**L**). Immunofluorescence staining with anti-human COL7 monoclonal antibody, LH7.2, fluorescein isothiocyanate, green (**C**, **F**, **I**, **L**, and **O**). White arrowheads, basement membrane zone; asterisks, a blister cavity. Dotted lines demarcate the skin surface. Scale bars = 50 μ m. **P:** Immunoblot analysis for human COL7 in the whole-skin samples from the wild-type and the three rescued mouse lines. Human COL7 protein expression was confirmed in the whole skin of all three lines of rescued mice (COL7^{m-/-}, K14-h+, COL7^{m-/-}, col1-h+, and COL7^{m-/-}, CMV-h+) but not in the wild-type mice. From the density of immunoblot bands, amounts of human COL7 expressed in the whole-skin samples were the greatest in COL7^{m-/-}, col1-h+ line among the three lines of rescued mice. The other two rescued lines, COL7^{m-/-}, K14-h+ and COL7^{m-/-}, CMV-h+, expressed roughly similar amounts of human COL7.

COL7^{m-/-}, col1-h⁺, and COL7^{m-/-}, CMV-h⁺) showed normal appearance at birth, and no DEB phenotype was observed (Figure 3, A, D, G, J, and M). Remarkably, all of the rescued mice (COL7^{m-/-}, K14-h⁺, COL7^{m-/-}, col1-h⁺, and COL7^{m-/-}, CMV-h⁺) exhibited reproductive ability despite the fact that the original COL7^{m-/-} mice were lethal and unable to reproduce. All of these rescued mice had at least a 1-year lifespan, similar to that of wild-type mice. We could not detect any blistering, even on a histological scale (Figure 3, B, E, H, K, and N) and immunofluorescence study using LH7.2 showed positive linear staining of COL7 along the BMZ in all three lines of rescued mice (COL7^{m-/-}, K14-h⁺, COL7^{m-/-}, col1-h⁺, and COL7^{m-/-}, CMV-h⁺) (Figure 3, C, F, I, L, and O). The pattern of positive staining was essentially identical to that in the respective original transgenic mice (Figure 2, A–H). Immunoblot analysis for human COL7 in the whole-skin samples from the transgenic rescued mice confirmed that human COL7 protein was expressed in the whole skin of all three lines of rescued mice (COL7^{m-/-}, K14-h⁺, COL7^{m-/-}, col1-h⁺, and COL7^{m-/-}, CMV-h⁺) (Figure 3P). The thicknesses of the immunoblot bands suggested that, in the whole-skin samples, COL7^{m-/-}, col1-h⁺ expressed the most human COL7 among the three lines of rescued mice, and the other two rescued lines, COL7^{m-/-}, CMV-h⁺ and COL7^{m-/-}, K14-h⁺, produced similarly less human COL7.

Electron microscopy of the skin showed newly formed anchoring fibrils in the sublamina densa area in all of the rescued mice (COL7^{m-/-}, K14-h⁺, COL7^{m-/-}, col1-h⁺, and COL7^{m-/-}, CMV-h⁺) (Figure 4, A, C, E, G, and I). Semiquantitative morphometric analysis of numbers of anchoring fibrils on electron microscopic images revealed the anchoring fibril density in each mouse line as follows (mean $\pm$ SD number of anchoring fibrils/ μ m): wild-type COL7^{m+/+}, 3.41 ± 0.43 ; COL7^{m-/-}, 0.00 ± 0.00 ; COL7^{m-/-}, K14-h⁺, 2.60 ± 0.46 ; COL7^{m-/-}, col1-h⁺, 2.85 ± 0.39 ; and COL7^{m-/-}, CMV-h⁺, 3.09 ± 0.30 (Figure 4K). Immunoelectron microscopic analysis of the skin obtained from each line of rescued mice revealed that LH7.2-labeled gold particles were localized in the lamina densa of the BMZ (Figure 4, B, D, F, H, and J). The epitopes of LH7.2 monoclonal antibody are known to react to the NC-1 domain of COL7, which is known to be located along the lamina densa.¹² The results suggest that COL7 synthesized from transgenes functioned correctly, irrespective of whether it originated from fibroblasts or from keratinocytes.

Generation of an Exact DEB Model Mice Carrying Human COL7A1 Mutation

In the course of cloning experiments, we obtained several mutant COL7A1 clones that demonstrated abnormal COL7A1 expression. Subsequent sequence analysis revealed that one of those clones had a c.7528delG mutation in the COL7A1 cDNA. We then constructed an expression vector of the mutated COL7A1 under the K14 promoter and generated the transgenic mice. Next, we crossed these mutant COL7^{K14-Δh} transgenic mice with COL7^{m+/+} het-

Figure 4. Transgene products, which are human COL7 molecules, correctly form anchoring fibrils in the rescued mice. Electron microscopy (EM) (A, C, E, G, and I) demonstrated intact anchoring fibrils (black arrowheads) in the sublamina densa area in a wild-type mouse (A). In contrast, the COL7^{m-/-} mouse had no anchoring fibrils (C). In all three rescued mice lines (keratinocyte-targeted rescued COL7^{m-/-}, K14-h⁺ [E], fibroblast-targeted rescued COL7^{m-/-}, col1-h⁺ [G], and ubiquitous CMV promoter-driven rescued COL7^{m-/-}, CMV-h⁺ [I]), anchoring fibril formation was restored in the sublamina densa area (black arrowheads). Immunoelectron microscopy (IEM) using LH7.2 (B, D, F, H, and J) revealed no human COL7 labeling (gold particle) in intact anchoring fibrils (black arrowheads) of a wild-type mouse (B). The COL7^{m-/-} mouse had neither anchoring fibrils nor human COL7 labeling (D). Human COL7 (immunogold particles, white arrowheads) was localized in the lamina densa of the basement membrane zone in all three rescued mice: keratinocyte-targeted rescued COL7^{m-/-}, K14-h⁺ (F), fibroblast-targeted rescued COL7^{m-/-}, col1-h⁺ (H), and ubiquitous CMV promoter-driven rescued COL7^{m-/-}, CMV-h⁺ (J). Black arrowheads, anchoring fibrils; white arrowheads, human COL7 labeling (immunogold particles); LD, lamina densa. Scale bars = 200 nm. K: Semiquantitative morphometric analysis of anchoring fibril density. Anchoring fibril density was highest in ubiquitous CMV promoter-driven rescued COL7^{m-/-}, CMV-h⁺, second highest in fibroblast-targeted rescued COL7^{m-/-}, col1-h⁺, and lowest in keratinocyte-targeted rescued COL7^{m-/-}, K14-h⁺ mice, among the three lines of rescued mice, although no statistically significant difference was observed between any combination of the three lines.

Figure 5. DEB model mice carrying a human *COL7A1* mutation precisely reproduce the DEB phenotype. **A–E:** *Col7a1* knockout mice rescued by full-length human *COL7A1* (*COL7^{m/-}, K14^{h+}*) are clinically normal. Forepaws of *COL7^{m/-}, K14^{h+}* mice at 14 days (**C**), 30 days (**D**), and 60 days of age (**E**). **F–J:** *Col7a1* knockout mice rescued by mutated human *COL7A1* (*COL7^{m/-}, K14^{Δh+}*) show gradual development of mild alopecia (**F**, arrowhead), yellowish dental caries (**G**, arrowhead), and fusion of the paw digits (**I** and **J**, arrowheads), corresponding to the clinical phenotype of human DEB. (**K** and **M**: A 2-year-old male patient harboring *COL7A1* mutations, c.[5818delC] + p.[Gly2623Ser].¹³ **L**: A 10-year-old female patient whose mutations were unidentified. The diagnosis was confirmed by ultrastructural observation and immunofluorescence studies. **N**: A 15-year-old male patient with p.[Gly2576Arg] + [Glu2858X].¹⁴ **O**: A 51-year-old female patient harboring p.[Gly1815Arg] + c.[5818delC].¹⁵) Forepaws of *COL7^{m/-}, K14^{Δh+}* mice were documented at 14 days (**H**, scarring, arrowhead), 30 days (**I**, mild fusion), and 60 days of age (**J**, complete fusion).

erozygous mice to obtain mutant *COL7^{m/-}, K14^{Δh+}* mice. We then performed transgenic rescue experiments by intercrossing these mice (*COL7^{m/-}, K14^{Δh+}*) and obtained *COL7^{m/-}, K14^{Δh+}* mice. Immunoblot analysis on epidermal extract samples from *COL7^{m/-}, K14^{Δh+}* mice confirmed the expression of short, truncated human COL7 derived from mutant *COL7A1* (data not shown).

From birth, the *COL7^{m/-}, K14^{Δh+}* mice were indistinguishable from their wild-type littermates and showed no blistering, not even on the paws, despite the fact that hemorrhagic bullae are always found in *COL7^{m/-}* mice. The growth of the human mutant-rescued mice (*COL7^{m/-}, K14^{Δh+}*) was retarded, however, compared with that of their wild-type littermates. Interestingly, the *COL7^{m/-}, K14^{Δh+}* mice gradually developed the DEB phenotype, including nail dystrophy, scarring on the paws, fusion of the digits, yellowish dental caries, and mild alopecia, characteristic features of human RDEB (Figure 5, K–O).^{13–15} It was difficult to distinguish the alopecia seen in the *COL7^{m/-}, K14^{Δh+}* mice from barbarism only from clinical appearance. However, the penetration of the alopecia is almost 100% in the *COL7^{m/-}, K14^{Δh+}* mice, whereas only a few wild-type lit-

termates that were kept in the same condition showed barbarism. Thus, this alopecia was considered to be a feature specific to the *COL7^{m/-}, K14^{Δh+}* mice. These DEB clinical phenotypic manifestations were evident at 2 months of age (Figure 5, F–J). The clinical course of the forepaws showed a phenotype that is very characteristic of DEB. The *COL7^{m/-}, K14^{Δh+}* mice showed no blistering at birth, yet there was scarring of the forepaws 2 weeks later (Figure 5H). By 1 month, the paws had become mildly fused (Figure 5I), and complete fusion of paws (mitten deformity) was observed at 2 months (Figure 5J). The growth of the full-length human gene-rescued mice (*COL7^{m/-}, K14^{h+}*) did not differ notably from that of the wild-type mice (Figure 5, A–E). During histopathological investigation, although clinically detectable blistering was not observed, we demonstrated microblistering along the dermal-epidermal junction in these mice (*COL7^{m/-}, K14^{Δh+}*) by histopathological analysis (Figure 6, A and C). Immunofluorescence analysis showed immunoreactivity of human COL7 in the BMZ of the *COL7^{m/-}, K14^{Δh+}* mice (Figure 6, B and D). Most of the human mutant mice had about a 6-month lifespan (Figure 5,

Figure 6. The humanized DEB model mouse shows subepidermal blistering with deposition of mutant human COL7 at the dermoepidermal junction. **A:** A $COL7^{m-/-}, K14^{h+}$ mouse has histopathologically normal skin. **C:** A $COL7^{m-/-}, K14^{\Delta h+}$ mouse shows subepidermal blistering (asterisks). **B** and **D:** Immunofluorescence study using anti-human COL7 antibody, LH7.2, reveals positive linear staining within the BMZ (arrowheads), corresponding to normal and mutant human COL7 in a $COL7^{m-/-}, K14^{h+}$ mouse and a $COL7^{m-/-}, K14^{\Delta h+}$ mouse, respectively. Dotted lines demarcate the skin surface. Scale bars = 50 μ m.

F-J). Thus, the clinical manifestations of human DEB were reproduced in the mouse by corrective transfer of human mutated *COL7A1* gene.

Discussion

COL7 is a major component of anchoring fibril loop structures beneath the epidermal basement membrane.^{12,16} Previous studies have indicated that epidermal keratinocytes are the primary source of COL7 in developing human skin.^{5,6} Thus, epidermal keratinocytes have been the main focus in the development of corrective gene therapies for DEB caused by *COL7A1* mutations. However, we recently showed that gene-transferred fibroblasts can supply a larger proportion of COL7 to the new dermal-epidermal junction as efficiently as gene-transferred keratinocytes.¹⁷ Moreover, fibroblasts are more robust and less susceptible to growth arrest and differentiation than are epidermal keratinocytes.⁶ Our study is the first *in vivo* study to show that keratinocytes and fibroblasts, through direct comparative studies, are both feasible targets for DEB gene therapy. In addition, this study can be extended to other basement membrane proteins, and fibroblasts may provide those proteins from the dermis toward the epidermis.

We first generated several transgenic mice with COL7 expression under the control of each of the following promoters: K14, *col1a2*, and CMV. We have shown that COL7 expression from either keratinocytes or dermal fibroblasts can be fully integrated into the epidermal BMZ *in vivo*. We have also shown that expression of COL7 by either keratinocytes or fibroblasts can successfully rescue $COL7^{m-/-}$ mice.³ Consequently, the rescued mice ($COL7^{m-/-}, K14^{h+}$, $COL7^{m-/-}, col1^{h+}$, and $COL7^{m-/-}, CMV^{h+}$) show expression of human COL7 under the control of the different pro-

motors. These three different rescued mouse lines show no evidence of the DEB phenotype, and their reproductive ability was restored. Ultrastructurally, newly formed anchoring fibrils were present, and the NC-1 domain of COL7 localized precisely in the lamina densa of the BMZ in the rescued mice. Collectively, these results provide future prospects for corrective gene therapy for DEB.

Generally speaking, the nature of promoters used in transgenes does not always define the amount of transgene expression in transgenic mice. In the present study, immunofluorescence and immunoblot analysis suggested that fibroblast-targeted rescued $COL7^{m-/-}, col1^{h+}$ mice expressed more human COL7 than that expressed in ubiquitous CMV promoter-driven rescued $COL7^{m-/-}, CMV^{h+}$ mice and keratinocyte-targeted rescued $COL7^{m-/-}, K14^{h+}$ mice. Interestingly, in contrast, semiquantitative morphometric analysis of anchoring fibril density revealed that anchoring fibril density was highest in the ubiquitous CMV promoter-driven rescued $COL7^{m-/-}, CMV^{h+}$ mice, among the three lines of rescued mice, although no statistically significant difference was confirmed. We cannot explain the exact mechanism behind this discrepancy. In ubiquitous CMV promoter-driven rescued $COL7^{m-/-}, CMV^{h+}$ skin, COL7 is produced by both fibroblasts and keratinocytes, similar to the physiological manner of COL7 expression. Thus, we speculate that, in $COL7^{m-/-}, CMV^{h+}$ skin, COL7 peptides might be more efficiently assembled to form anchoring fibrils, even if less protein is expressed than in the $COL7^{m-/-}, col1^{h+}$ or $COL7^{m-/-}, K14^{h+}$ skin in which COL7 is expressed only by fibroblasts or keratinocytes, respectively.

The mice developed in this study can also provide a useful model for immunobullous diseases involving COL7. Recently, we succeeded in generating a bullous pemphigoid model.¹⁸ Passive transfer of bullous pemphigoid autoantibodies into wild-type mice has failed to induce skin lesions, because of differences between humans and mice in the amino acid sequence of the pathogenic epitope of the autoantigen, COL7.¹⁹ We injected the patients' autoantibody into murine COL7 knockout mice that had been rescued by the expression of the human autoantigen. This resulted in successful reactions by autoantigens and autoantibodies, thereby producing the bullous pemphigoid phenotype. Epidermolysis bullosa acquisita is an autoimmune blistering disorder, and the patients' autoantibodies react to COL7. Therefore, the rescued mice with humanized COL7 that we produced should be useful in future research on epidermolysis bullosa acquisita as well.

Another interesting aspect of the present study is that we were able to develop $COL7^{K14-\Delta h+}$ transgenic mice with human *COL7A1* cDNA containing the mutation c.7528delG. The $COL7^{K14-\Delta h+}$ transgenic mice and the $COL7^{m-/-}, K14^{\Delta h+}$ rescued mice showed positive human COL7 staining at the BMZ, indicating that COL7 without the NC-2 domain can still form a triple helix and be secreted by keratinocytes. The characteristic component of all collagens is the triple helix formed by three subunits, and its assembly is based on the repetition of the Gly-X-Y repeats. It has been suggested that a zipper-like mechanism of triple helix formation starts from the C

terminus toward the N terminus in collagens I and IV^{20–22} and from the N-terminal to the C-terminal direction in epidermal type XVII collagen.²³ Our experiments using the genetically engineered mouse model suggest that the N- to C-terminal mechanism of triple helix formation is also possible for COL7. However, lack of the NC-2 domain, which is critical for antiparallel-dimer formation, might cause partial and weak immunoreactivity of human COL7 in the BMZ of COL7^{m-/–}, K14-Δh^{+/+} mice. This study demonstrates the importance of the NC-2 domain in COL7 formation and assembly *in vivo*.

Of importance, we have generated a mouse model of DEB that allows for long-term studies that were not possible with the previously generated neonatal lethal COL7^{m-/–} *col7a1* knockout mice. A surviving DEB mouse model (the mouse COL7 hypomorphic mouse) that was recently reported expresses mouse COL7 at approximately 10% of normal levels.⁴ These mice could survive longer than *Col7a1* knockout mouse (COL7^{m-/–}) and present clinical phenotypes (mitten hands and feet) similar to those of human DEB. The phenotypes of these model mice were produced from the gene-engineered mouse COL7 gene using a hypomorphic technique. These mice had a high mortality rate (67%) within 28 days without a change to a liquid diet consisting of infant milk. On the contrary, our novel mouse models of RDEB were generated by completely different methodology using a mutated human *COL7A1* gene, and the mouse could survive longer without use of a liquid diet. Surprisingly, our original DEB model mouse is very similar to humans not only in terms of clinical manifestations but also in terms of the genetic background. In fact, the COL7^{m-/–}, K14-Δh^{+/+} mice demonstrated nail dystrophy, scarring on the paws, fusion of the digits, yellowish dental caries, and mild alopecia, even in the absence of overt blistering. The previous *col7a1* knockout COL7^{m-/–} mice developed spontaneous blistering soon after birth and died within several days.³ Thus, COL7^{m-/–} mice have not been available for long-term experiments. In this study, the production of rescued mice with mutated COL7A1 (COL7^{m-/–}, K14-Δh^{+/+}) has given us a surviving model of DEB. This model has great potential for future research into the pathomechanisms of DEB, wound healing, the development of squamous cell carcinomas, and the development of molecular therapies for patients with DEB.

Although we used cDNA with the mutation c.7528delG, which causes a premature stop termination codon (PTC), the consequences of the PTC mutation in the *COL7A1* cDNA are different from those in the *COL7A1* gene. Genomic PTC mutations are subject to nonsense-mediated mRNA decay, resulting in mRNA degradation in some instances. In the literature, genomic PTC mutations in *COL7A1* were previously reported to result in nonsense-mediated mRNA decay and absence of COL7 protein synthesis in severe generalized cases of RDEB.^{24,25} Whether a genomic PTC mutation leads to nonsense-mediated mRNA decay depends on the mutation site.²⁶ In contrast, the PTC mutation in cDNA does not lead to mRNA decay and is thought to generate a truncated protein. In fact, we confirmed the expression of human COL7 derived from human mutant *COL7A1* in the COL7^{m-/–}, K14-Δh^{+/+} mice by immunoblot analysis (data

not shown) and immunofluorescence staining (Figure 6D) in the present study. Approximately 300 distinct *COL7A1* mutations have been identified in patients with DEB around the world, and the clinical features, severity, prognosis, and response to treatment vary depending on the specific mutation.^{15,24,27–31} Our understanding of how specific mutations produce differing clinical presentations and prognoses is limited. We believe that our systems have the advantage of being able to use human genes. Because the COL7 gene is almost 30 kb in size, introduction of the gene with PTC mutation might be impractical. However, if we generate the same mouse models with the patient-specific missense mutations in the cDNA or with the patient-specific PTC mutation in partial genomic DNA, which was inserted in the cDNA, then they might be useful for evaluating the prognosis of each patient with a certain mutation and for developing a mutation-specific treatment. This strategy could be extended to the development of therapies tailored to other, currently intractable inherited diseases.

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