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Targeted inactivation of the type VII collagen gene (Col7a1) in mice results in severe blistering phenotype: a model for recessive dystrophic epidermolysis bullosa

Seppo Heinonen[1,*], Minna Männikkö[1,*], John F. Klement[1,4,*], Diana Whitaker-Menezes[2], George F. Murphy[2] and Jouni Uitto[1,3,4,‡]

Departments of [1]Dermatology and Cutaneous Biology, [2]Pathology, Anatomy and Cell Biology, and [3]Biochemistry and Molecular Pharmacology, Jefferson Medical College, and [4]Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia, PA 19107, USA

*These authors contributed equally to this study

‡Author for correspondence (e-mail: jouni.uitto@mail.tju.edu)

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SUMMARY

Dystrophic forms of epidermolysis bullosa (DEB) are associated with mutations in the type VII collagen gene (Col7a1) which encodes the major component of anchoring fibrils. To develop a DEB animal model, type VII collagen deficient mice were generated by targeted homologous recombination. The targeting vector replaced exons 46-69 of Col7a1 with the neomycin-resistance gene, in reverse transcriptional orientation, resulting in elimination of most of the collagenous domain 1. Col7a1 heterozygous (+/–) mice were phenotypically normal. Mating of Col7a1 +/– mice revealed that Col7a1 null (–/–) mice, which were born with extensive cutaneous blistering, died during the first two weeks of life probably due to complications arising from the blistering. Transmission electron microscopy revealed subepidermal blistering below the lamina densa and absence of anchoring fibrils. Immunohistochemical staining with anti-human type VII collagen antibody stained the dermal-epidermal junction in control mice, but did not stain the skin of Col7a1 null mice. Collectively, the DEB mice recapitulate the clinical, genetic, immunohistochemical and ultrastructural characteristics of recessive DEB in humans. These mice provide an animal model to study the pathomechanisms of DEB and serve as a system to test therapeutic approaches, including gene replacement, towards the cure of this devastating skin disease.

Key words: Type VII collagen, Epidermolysis bullosa, Dermo-epidermal junction, Genetic skin disease, Gene therapy

INTRODUCTION

Epidermolysis bullosa (EB) is a group of heritable mechano-bullous disorders characterized by fragility of the skin and mucous membranes. Traditionally, EB has been divided into three broad categories based on the level of tissue separation within the cutaneous basement membrane zone (BMZ) (Christiano and Uitto, 1996a; Fine et al., 1999; Uitto et al., 1997). One of these subsets, the dystrophic forms of EB (DEB), is inherited in either an autosomal dominant (DDEB) or autosomal recessive (RDEB) fashion. DEB is characterized by tissue separation below the lamina densa, at the dermal side of the dermal-epidermal basement membrane where anchoring fibrils are located (Christiano and Uitto, 1996b). Ultrastructurally, the non-blistered skin of patients with DEB is characterized by abnormalities in the anchoring fibrils, attachment structures extending from the lower portion of the dermal-epidermal basement membrane to the underlying dermis (Tidman and Eady, 1985). These structures can be morphologically altered, reduced in number, or entirely absent in DEB patients' skin.

Early biochemical evidence indicated that type VII collagen is the major, if not the exclusive, protein component of the anchoring fibrils (Sakai et al., 1986; Keene et al., 1987). Type VII collagen, as all collagen molecules (Kivirikko, 1993), consists of three polypeptide chains, $[\text{pro}\alpha1(\text{VII})]_3$. The central portion of the trimer type VII collagen molecule consists of a collagenous segment, with repeating Gly-Xaa-Yaa amino acid sequence, which is interrupted by imperfections, including a 39-amino acid 'hinge' region in the middle of the collagenous sequence (Christiano et al., 1994a). Thus, the triple-helical region of type VII collagen is divided into two segments, the collagenous domains 1 and 2, of approximately equal size. The collagenous domains are flanked by a large, ~145 kDa, non-collagenous amino-terminal globular domain (NC1) and a smaller, ~20 kDa, carboxy-terminal globular domain (NC2)

(Christiano et al., 1994a). Type VII collagen is synthesized primarily by epidermal keratinocytes in vivo, although other cell types, including dermal fibroblasts, do express the gene at lower levels (Ryynänen et al., 1992; König et al., 1992). Following secretion, type VII collagen molecules form anti-parallel dimers with overlapping carboxy-terminal ends, part of the NC2 domain is proteolytically removed, and the association of the molecules is stabilized by intermolecular disulfide bonding. Subsequently, a large number of type VII collagen dimer molecules laterally assemble into anchoring fibrils which extend from the lamina densa of the basement membrane to the upper papillary dermis, thus stabilizing the association of epidermis to the underlying dermis (Burgeson, 1993).

Observations of anchoring fibril abnormalities in DEB suggested that type VII collagen, and the corresponding human gene, COL7A1, are the candidate protein/gene systems harboring the mutations in this disease (Uitto and Christiano, 1992). This suggestion was supported by immunohistochemical studies which revealed attenuated or absent immunofluorescence staining of the BMZ with antibodies recognizing type VII collagen epitopes (Bruckner-Tuderman, 1991). Upon cloning of human type VII collagen cDNAs and mapping of the corresponding chromosomal locus (Parente et al., 1991), this notion was further supported by demonstration of a strong genetic linkage between COL7A1 and the disease gene in both dominantly and recessively inherited forms of DEB (Ryynänen et al., 1991; Hovnanian et al., 1992). The combined LOD score in families with DDEB (n=14) and RDEB (n=54) was Z = 41.4 and Z = 10.6, respectively, with no evidence of recombination (for summary, see Uitto and Christiano, 1994). Upon elucidation of the intron-exon organization of the entire human COL7A1 gene (Christiano et al., 1994b), different mutation detection strategies were developed, identifying a number of distinct genetic lesions in COL7A1 in DEB (Hovnanian et al., 1997; Järvikallio et al., 1997; Pulkkinen and Uitto, 1999).

Although DEB has been shown to result from mutations in COL7A1, relatively little is known of the mechanistic consequences of the mutations in relation to blistering. Furthermore, no efficient treatment, besides avoidance of trauma and infections, is currently available for this group of blistering diseases. To facilitate our understanding of DEB, a transgenic mouse deficient in type VII collagen was developed by gene targeting strategy. Clinical, histochemical, and ultrastructural observations reported here demonstrate that this mouse serves as an excellent model for RDEB.

MATERIALS AND METHODS

Targeting vector construction

P1 recombinant phage containing mouse strain 129Sv Col7a1 gene fragments were obtained from Genome Systems, Inc. (St Louis, MO), as previously described (Kivirikko et al., 1996). *Bam*HI DNA fragments from these phage clones were randomly cloned into the *Bam*HI site of pBluescript SK(+) (Stratagene) and identified by colony hybridization with oligonucleotides from the Col7a1 gene (GenBank accession number U32177; Kivirikko et al., 1996), followed by DNA sequencing. Col7a1 *Bam*HI fragments of 1.8 kb (nucleotides 11928 to 13765) and 3.2 kb (nucleotides 19191 to 22436) were inserted into the *Not*I/*Xho*I and *Xba*I/*Eco*RI sites, respectively, of pPNT (Tybulecwicz et al., 1991). The final construct (pC7KO) has the phosphoglycerate kinase promoter/neomycin resistance gene (PGK-*neo*) in the opposite transcriptional orientation relative to the Col7a1 gene. Embryonic stem (ES) cells (W9.5; Stewart, 1993) were electroporated with 30 μg of *Not*I linearized pC7KO, plated, and double selected with G418 and ganciclovir, as described (Köntgen and Stewart, 1993). Southern hybridization with a PCR generated 3′ flanking probe (nucleotides 22655 to 24072) was used to identify ES cells having undergone Col7a1 homologous recombination. This probe will hybridize to a 7.9 kb *Eco*RV DNA fragment from Col7a1 and a 4.9 kb *Eco*RV DNA fragment from the recombined Col7a1 gene (Fig. 1). Of 288 G418/ganciclovir resistant clones, four clones were identified with the disrupted Col7a1; two ES clones were used to microinject C57BL6/J donor blastocysts generating chimeric mice as described (Stewart, 1993). One of the Col7a1 ES clones produced chimeric mice which appeared to be largely derived from the ES cells judging by the agouti coat color (see Results). The fidelity of recombination at the 5′ end of the Col7a1 null gene was confirmed by Southern analysis using the 1.8 kb *Bam*HI fragment (nucleotides 11928 to 13765) as probe and *Sph*I digestion of genomic DNA (see Fig. 1). The *Sph*I restriction enzyme digestion produced a 5.4 kb and a 3.0 kb *Sph*I DNA fragments from the wild-type and null Col7a1 alleles, respectively (not shown).

PCR screening of the mice

Template DNA for genomic PCR was isolated from tail tissue of the mice by standard methods (Sambrook et al., 1989). Three primers were used to screen for wild-type (+/+), heterozygote (+/–) and null (–/–) mice.

Primer 1: 5′ AGG TAT CAT ACT TCC TGG CAG A 3′;
Primer 2: 5′ AAG GCT ATC AAT ACT AGA ACC AG 3′;
Primer 3: 5′ CCT TCT TGA CCA GTT CTT CTG A 3′.

Primers 1 and 2 amplified a 650 bp region of normal type VII collagen, and primers 1 and 3 amplified a 490 bp region between the type VII collagen and the *neo* genes in separate reactions. PCR reactions were performed in 25 μl containing 50-100 ng of template, 50 ng of primers, 0.2 μM of each nucleotide, 1:10 vol. of 10× GenAmp buffer with 1.5 mM $MgCl_2$, and 1.5 U AmpliTaq DNA polymerase (Perkin Elmer). The amplification cycles were 94°C for 5 minutes, followed by 35 cycles of 94°C for 45 seconds; 57°C for 45 seconds; and 72°C for 45 seconds; followed by an additional extension for 8 minutes at 72°C.

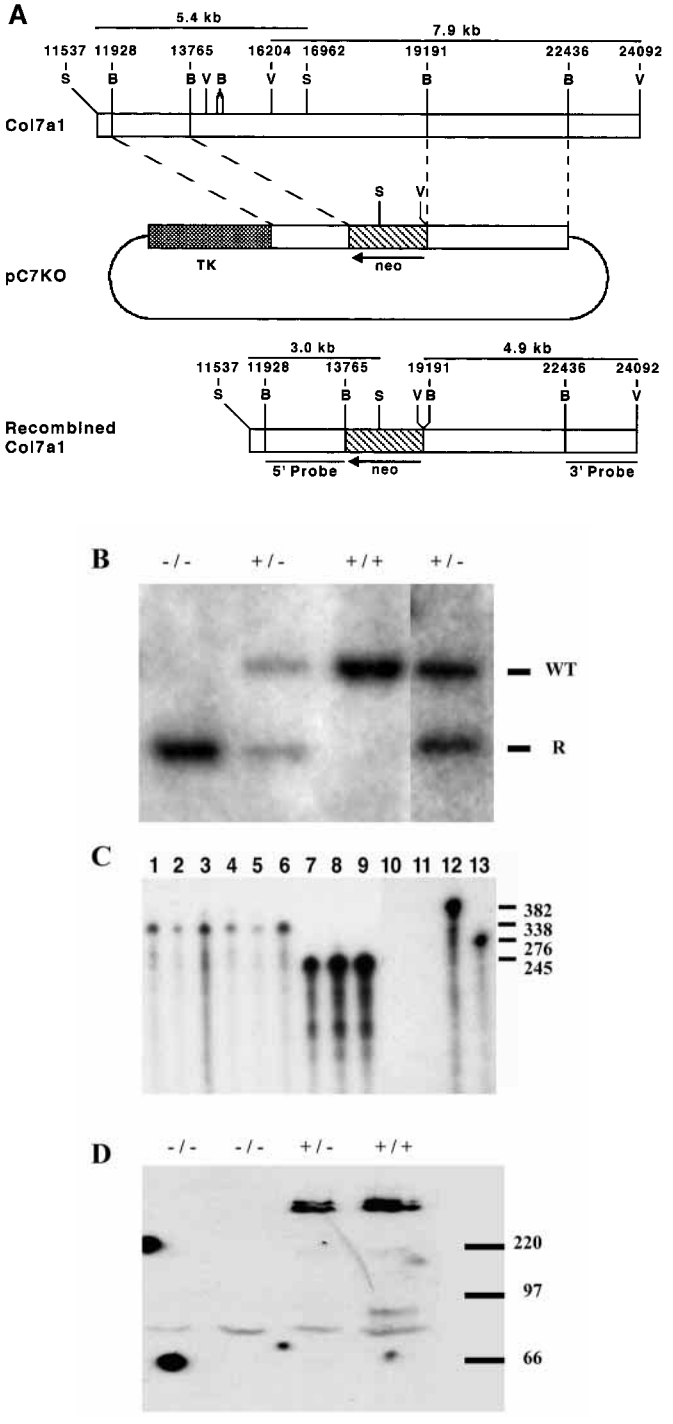
RNA isolation and RNase protection assay

RNA was isolated from Col7a1 +/+, +/–, and –/– mouse skin using Trizol Reagent (Gibco BRL Life Technologies). RNase protection assays were performed with 40 and 20 μg of total RNA using the RPA III kit (Ambion Inc.). T3 RNA polymerase-synthesized anti-sense probe (MAXIscript, Ambion Inc.) consisted of 338 nucleotides (nt) of mouse type VII collagen cDNA corresponding to positions +1 to +333 (GenBank accession number U32107), 5 nt of flanking Col7a1 5′ untranslated sequence, and flanking sequences from pBluescript KS+ (Stratagene). Thus, the entire probe is 382 nt and the RNase protected fragment is expected to be 338 nt. Linearized pTRI-actin (mouse) DNA (RPA III kit, Ambion Inc.) was transcribed with T3 RNA polymerase, and the labeled transcript was used as a control.

Western blotting

Whole tissue extracts were prepared by homogenizing mouse skin in lysis buffer (8 M urea, 2% SDS, 0.1 M DTT, 0.1 M Tris-HCl, pH 6.8, and 0.25 mM PMSF). Lysates were incubated for 3 minutes at room temperature with shaking, and centrifuged twice for 10 minutes (12,000 ***g*** at room temperature) to remove any insoluble material. Soluble proteins of the extract were separated on 6% SDS-polyacrylamide gel and transferred to nitrocellulose (ECL Nitrocellulose, Amersham Corp.). The membrane was incubated for 2 hours in blocking solution (5% w/v fat-free milk powder, 0.1% w/v

BSA, and 1% v/v normal goat serum in PBS). The primary antibody, rabbit anti-human NC1 domain of type VII collagen (kindly provided by Drs Mei Chen and David Woodley), diluted 1:20,000 in blocking buffer, was incubated for 1 hour at room temperature. The membrane was washed three times (once for 15 minutes, and twice for 5 minutes) in 1%, v/v, Tween-20, 0.1%, w/v, BSA in PBS, and then incubated for 30 minutes with secondary antibody, anti-rabbit immunoglobulins conjugated with horseradish peroxidase (DAKO), 1:2000 dilution in blocking buffer. The membranes were washed again as described above. Binding of the secondary antibody was detected with the ECL system (Amersham Corp.)



Immunoperoxidase staining

Acetone-fixed cryosections (5 μm) were stained using a three-step immunoperoxidase method employing avidin-biotin horseradish peroxidase (Vector Laboratories, Burlingame, CA). Briefly, tissue sections were incubated with either rabbit polyclonal antibody to human type VII collagen described above or normal rabbit immunoglobulins for 1 hour at room temperature. After washing in PBS, biotinylated goat anti-rabbit antibody was applied for 30 minutes as the secondary layer, followed by an avidin-biotin horseradish peroxidase complex for 30 minutes at room temperature. Immunoreactivity was then revealed using 3, 3′ diaminobenzidine (Sigma Chemical, St Louis, MO) as the substrate, and sections were lightly counterstained with Gill's hematoxylin no. 1 (Fisher Scientific, Malvern, PA).

Light and electron microscopy

For light microscopy, tissue samples were immediately placed in 10% phosphate-buffered formalin and allowed to fix for at least 24 hours before processing. The samples were then dehydrated through graded alcohols and xylene and embedded in paraffin. Sections were cut at 6 μm and stained with hematoxylin and eosin.

For electron microscopy, samples were immediately placed in Karnovsky's II fixative (Karnovsky, 1965) and stored at 4°C until processing. They were then washed with 0.1 M cacodylate buffer, pH 7.4, post-fixed with 2% osmium tetroxide, dehydrated through graded ethanols with propylene oxide as the final step, and embedded in Taab Epon 812 (Marivac Ltd, Nova Scotia, Canada). One micron sections were stained with 0.5% toluidine blue in 0.5% sodium borate and areas were evaluated for further analyses. Ultrathin sections were cut on a Leica Ultracut UCT ultramicrotome, post-stained with uranyl acetate followed by lead citrate, and viewed on a Hitachi H7000 electron microscope.

Fig. 1. (A) Schematic diagram of *Col7a1* gene-targeting strategy. A portion of the murine Col7a1 gene from bases 11537 to 24092 (GenBank accession number #U32177) is shown at the top, pC7KO is depicted in the middle, and the recombined Col7a1 locus is shown at the bottom. The diagnostic *Eco*RV DNA restriction fragments, with their sizes, are indicated; the positions of the 3′ and 5′ probes are shown; and the transcriptional orientation of the *neo* gene is depicted by the arrow. Abbreviations: TK, Herpes Simplex virus thymidine kinase gene; neo, phosphoglycerate kinase promoter/neomycin resistance gene; B, *Bam*HI; V, *Eco*RV, S, *Sph*I. (B) Southern analysis of transgenic mice. Note the presence of a 7.9 kb *Eco*RV DNA fragment in the wild-type animals (WT); the 4.9 kb *Eco*RV DNA fragment is indicative of the recombined Col7a1 gene (R). The most right-hand lane is an overexposure of a +/– lane, indicating the approximately equal intensities of the WT and R fragments. (C) RNase protection assay of Col7a1 null (lanes 1, 4, 7), heterozygous (lanes 2, 5, 8), and wild-type (lanes 3, 6, 9) mice. Lanes 1-3 represent 40 μg, and lanes 4-6 represent 20 μg of total skin RNA hybridized with Col7a1 antisense RNA probe producing a protected fragment of 338 nt after RNase digestion. Lanes 7-9 contain 10 μg of Col7a1 –/–, +/– and +/+ mouse skin RNA hybridized with pTRI mouse-actin control probe indicating an RNase protected fragment of 245 nt. Lanes 10 and 11 are yeast tRNA hybridized with Col7a1 and pTRI-actin control probes respectively. Unprotected Col7a1 (382 nt) and pTRI-actin (276 nt) probes are shown in lanes 12 and 13, respectively. (D) Western analysis of Col7a1 in skin protein extracts from wild-type (+/+), heterozygous (+/–), and null (–/–) mice performed using rabbit anti-human collagen VII antibody. Two polypeptides of approximately 300 kDa were detected using anti-collagen VII antibody and are within the range of expected sizes of unprocessed and partially processed collagen α1(VII) polypeptides. In the –/– protein samples the antibody failed to detect these 300 kDa bands indicating lack of type VII collagen in the skin of Col7a1/null mice. Protein molecular mass markers are indicated on the right of the figure.

RESULTS

Generation of mice with targeted inactivation of the Col7a1 gene

The Col7a1 gene in mice and humans consists of 118 exons, the largest number of exons in any gene characterized thus far (Christiano et al., 1994b; Kivirikko et al., 1996). The strategy to disrupt the Col7a1 gene in mice consisted of construction of a targeting vector which would delete a 5 kb segment spanning from exon 46 to exon 69 (Fig. 1A). This segment was replaced by the PKG-*neo* gene in the recombinant locus in reverse transcriptional orientation relative to Col7a1. The deletion removes a total of 372 codons within the central collagenous domain 1 of type VII collagen.

Injection of ES clones with the targeted allele into C57BL6/J blastocysts resulted in two male chimeras which showed germline transmission of the ES genome, as determined by the agouti coat color of the offspring. Southern analysis (Fig. 1B) revealed that ~50% of the agouti animals were heterozygous (+/–) for the targeted mutation. Col7a1 +/– animals were clinically normal and indistinguishable from the wild-type (+/+) littermates. Heterozygous mice were intercrossed to produce Col7a1 null (–/–) offspring. Thus far, 82 pups representing nine litters have been genotyped by Southern analysis and/or PCR amplification (see Materials and Methods). Among these animals, 17% were +/+, 54% were +/–, and 29% were –/–, suggesting Mendelian inheritance of the targeted mutation. Chi-square (χ^2) test indicated that the transgene distribution in the offspring did not statistically deviate from the expected Mendelian segregation (P>0.05). Furthermore, there was no evidence of excess embryonic lethality of animals.

Col7a1 –/– mice do not express type VII collagen in their skin

To demonstrate the consequences of the gene targeting at the mRNA and protein levels, skin from the offspring of Col7a1 +/– mice was examined by RNase protection assay and western analysis. First, RNase protection assays were performed with a 382 nt antisense probe containing 338 nt of the 5′ region of Col7a1 mRNA. An RNase protected fragment of 338 nt was observed with Col7a1 +/+, +/–, and –/– mouse skin total RNA (Fig. 1C). As the PGK-neo cassette was inserted downstream of the protected sequence, the results indicate that the endogenous Col7a1 gene promoter is functional in Col7a1 –/– mice. The pTRI-actin control probe of 276 nt revealed the expected protected RNA fragment of 245 nt with Col7a1 +/+, +/–, and –/– mouse RNA (Fig. 1C).

Secondly, western blot analysis of skin protein from normal +/+ and +/– mice revealed two closely migrating protein bands in the range of 300 kDa, the expected size of the α1(VII) polypeptides (Fig. 1D). The presence of two distinct proteins may reflect processing of these polypeptides that has been shown to take place in the skin (Bruckner-Tuderman et al., 1995). In contrast, western analysis of the skin from –/– mice failed to detect any proteins in the 300 kDa range (Fig. 1D). Furthermore, there was no evidence of a shortened polypeptide of ~163 kDa, which would be expected from the expression of the 5′ end of the mutant allele with a putative translation stop codon within the PGK-*neo* gene. Furthermore, no evidence of a polypeptide of ~263 kDa in case the *neo* gene had been spliced out in frame was noted. These data indicate that the targeted mutation results in a null allele of Col7a1.

Col7a1 –/– mice demonstrate neonatal skin blistering resembling the phenotype in RDEB patients

Col7a1 –/– mice demonstrated a striking phenotype noted at birth or shortly thereafter. Specifically, large blisters developed primarily on the ventral side of the animals often extending to cover the extremities, while the dorsal side developed blisters less frequently (Fig. 2A-C). Furthermore, the animals developed erosions on the feet (Fig. 2D,E), and blistering of the mucous membranes of the oral cavity was also noted in older pups (not shown). Most of the affected animals died within the first week of life from complications of the disease. A few animals survived beyond the first week of life and demonstrated normal hair growth which appeared to coincide with lessening of the blistering tendency in the skin, consistent with the suggestion that abundant hair growth ameliorates the blistering phenotype (Sundberg, 1994). These mice continued to develop oral blisters, and eventually most of them died before the age of 2 weeks. To date, only one mouse has

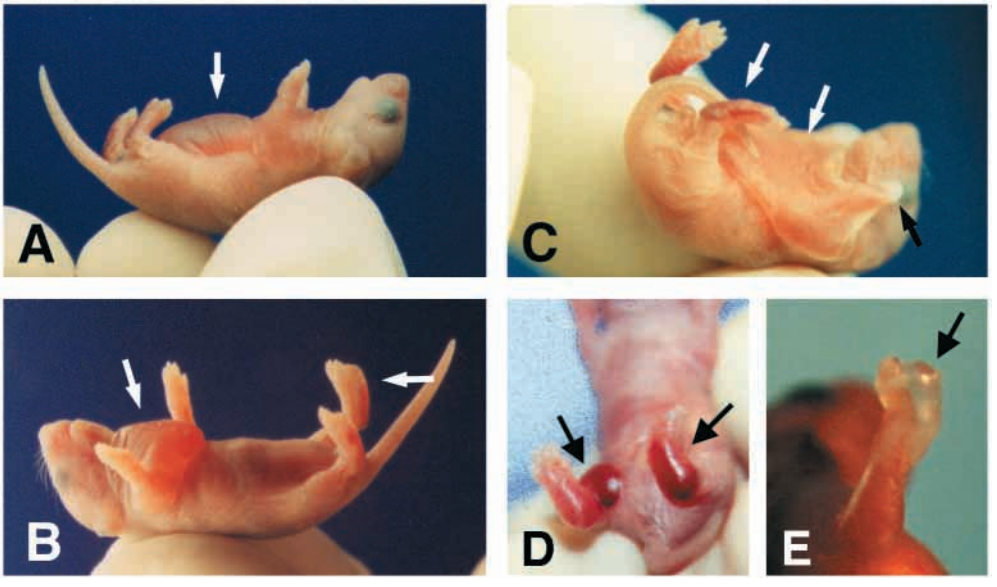


Fig. 2. Clinical presentation of mice deficient in type VII collagen. (A-C) Extensive blistering of the ventral skin (white arrows). Note in C (black arrow) that the blister encompasses the forepaw resulting in fusion of the digits. (D and E) Typical blisters seen on paws, (D) hemorrhagic blisters, (E) a clear blister.

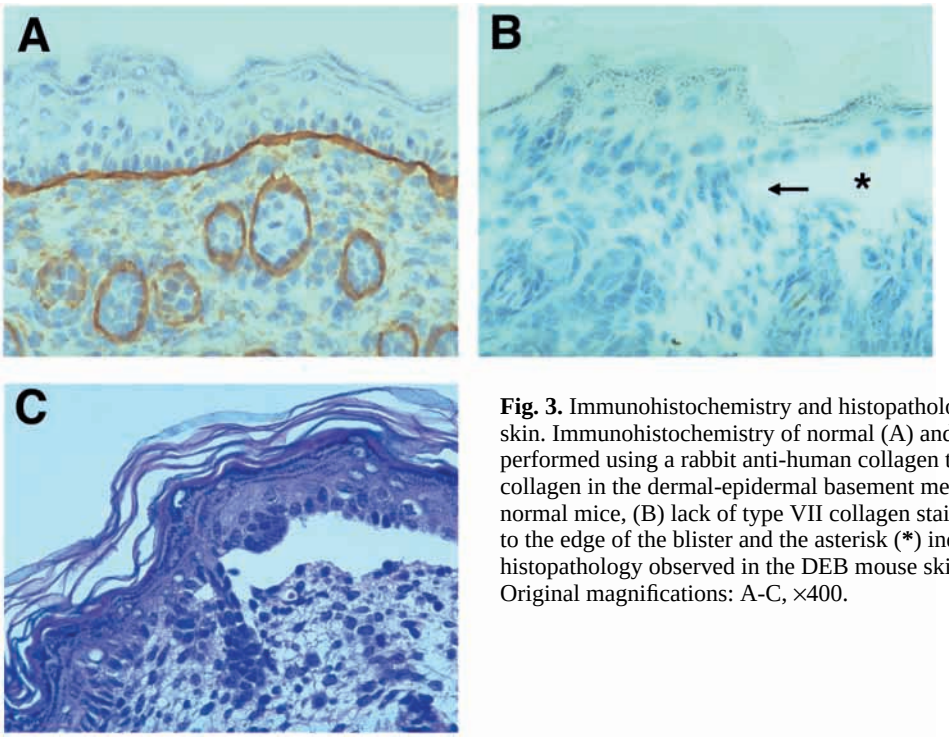


Fig. 3. Immunohistochemistry and histopathology of type VII collagen deficient mouse skin. Immunohistochemistry of normal (A) and type VII collagen deficient mouse skin (B) performed using a rabbit anti-human collagen type VII antibody. (A) Staining of type VII collagen in the dermal-epidermal basement membrane zone and around the hair follicles in normal mice, (B) lack of type VII collagen staining in a DEB mouse. In B the arrow points to the edge of the blister and the asterisk (*****) indicates the blister cavity. (C) The histopathology observed in the DEB mouse skin associated with a developing blister. Original magnifications: A-C, ×400.

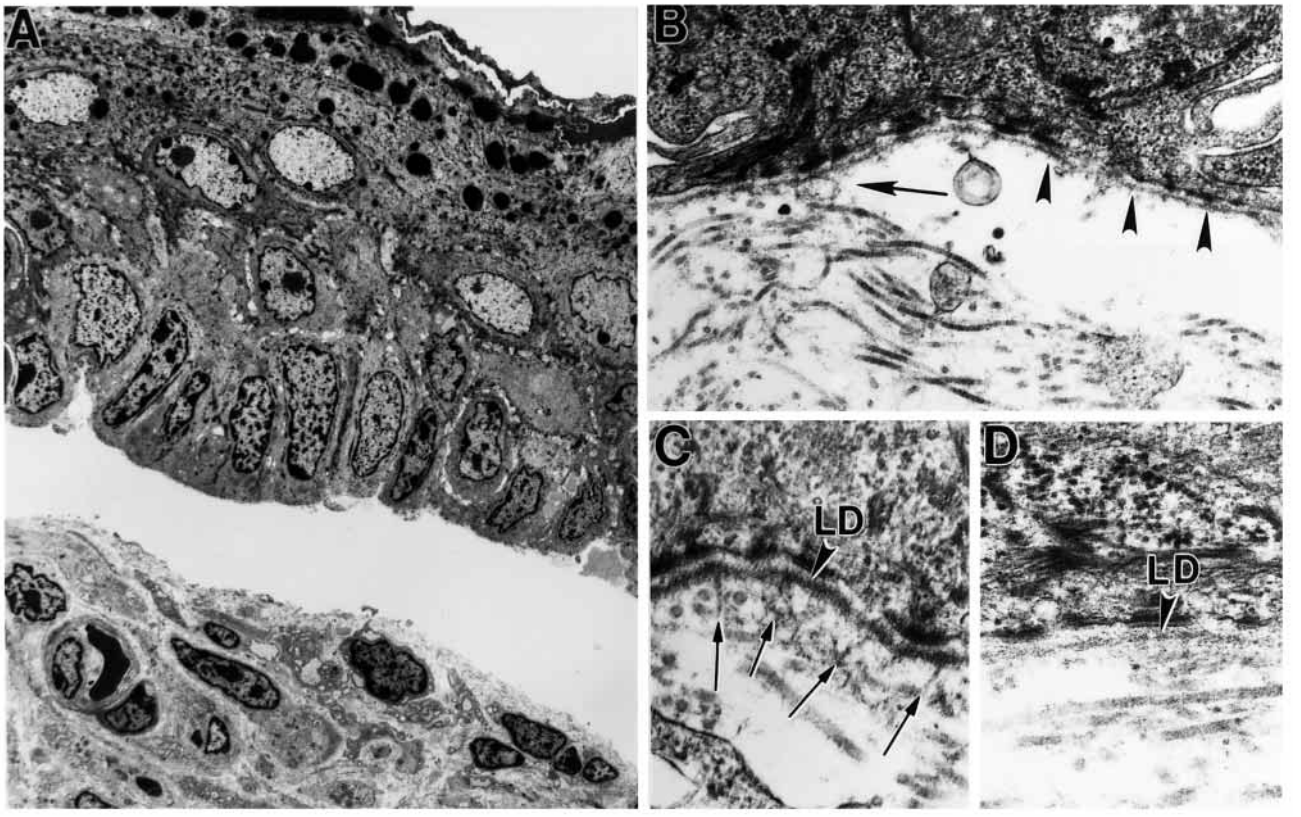


Fig. 4. Transmission electron microscopy of the skin of Col7a1 –/– mice. (A) Examination of the perilesional skin shows dermal-epidermal separation. (B) Higher magnification shows that the separation is below the lamina densa. The arrow points to the site of tissue separation and arrowheads indicate the lamina densa. (C) Examination of +/+ control mice reveals the presence of anchoring fibrils (arrows), which extend from the lamina densa (LD) to the underlying dermis. (D) The anchoring fibrils are entirely absent in the skin of Col7a1 –/– mice. Magnifications: A, ×2,000; B, ×23,250; C, ×40,500; D, ×40,000.

survived past three weeks, requiring extensive supportive care (see Discussion). The +/– mice were phenotypically indistinguishable from their +/+ littermates, showing no evidence of skin fragility.

Histopathologic examination of the perilesional ventral skin in –/– mice demonstrated tissue cleavage at the dermal-epidermal junction (Fig. 3C). Interestingly, histopathology of the dorsal skin of –/– mice, in the area which did not demonstrate clinically detectable blisters, showed focal dermal-epidermal tissue separation. Skin biopsy specimens were obtained from clinically uninvolved skin from DEB and wild-type mice for immunohistochemical staining with an antibody recognizing type VII collagen epitopes within the amino-terminal NC1 domain. In the skin of wild-type animals this antibody decorated the dermo-epidermal junction in a linear fashion (Fig. 3A). In contrast, the staining of skin from –/– mice was entirely negative, indicating the absence of type VII collagen (Fig. 3B).

Transmission electron microscopy (Fig. 4A) confirmed that the blister formation took place in the sub-lamina densa area at the dermal side of the cutaneous BMZ, the lamina densa being intact in the roof of the blister (Fig. 4B). Electron microscopy of the non-blistered skin in –/– mice (Fig. 4D) also revealed the absence of anchoring fibrils, which were readily detectable in both +/– and +/+ littermates (Fig. 4C). Thus, the skin of the Col7a1 –/– mice has pathologic features similar to those encountered in RDEB patients.

DISCUSSION

Clinically, DEB manifests with a spectrum of phenotypic severity, with considerable inter-familial heterogeneity (Christiano and Uitto, 1996a,b; Fine et al., 1999). The most severe, recessively inherited form of DEB, the Hallopeau-Siemens variant (HS-RDEB), manifests with extreme fragility of skin, noted at birth or shortly thereafter. The extensive blistering leads to scarring and fusion of the digits (pseudosyndactyly), associated with extracutaneous manifestations, such as oral blistering, esophageal strictures, and corneal erosions (Christiano and Uitto, 1996b). Later in life, development of multicentric, aggressively metastasizing squamous cell carcinomas can lead to premature demise of the individuals affected by DEB (see Christiano et al., 1999). In the milder, recessively inherited variants, so-called mitis forms of DEB, M-RDEB, blistering leads to less pronounced scarring with little or no tendency for pseudosyndactyly (Shimizu et al., 1996). However, the clinical phenotype of the recessively inherited forms presents with a continuum of severity, and some patients are classified as HS/M-RDEB. Dominantly inherited forms of DEB (DDEB) manifest with relatively mild blistering, primarily on the hands and feet, a phenotype clinically indistinguishable from the milder forms of M-RDEB. In spite of phenotypic variability, all recognized forms of DEB are associated with genetic abnormalities in the type VII collagen gene (COL7A1) (Järvikallio et al., 1997).

The mice developed in this study were clearly deficient in type VII collagen. Specifically, immunohistochemical staining with antibodies recognizing type VII collagen epitopes was entirely negative at the dermal-epidermal junction, and no expression of the shortened polypeptide could be detected by western analysis. Transmission electron microscopy confirmed the absence of anchoring fibrils and revealed sub-lamina densa blistering. As the heterozygote carriers of the transgene were clinically normal, this mouse mutation is clearly recessive, and these mice serve as a model for RDEB.

Although the total number of mutations disclosed in humans with different forms of DEB exceeds 100, relatively little information exists on genotype/phenotype correlations (Järvikallio et al., 1997; Pulkkinen and Uitto, 1999). However, certain general rules are beginning to emerge from examination of the mutation database. For example, in the majority of cases with the severe mutilating HS-RDEB, the genetic lesions consist of premature termination codon mutations (PTC) in both alleles, which predict synthesis of severely truncated type VII collagen polypeptides unable to assemble into functional anchoring fibrils (Uitto et al., 1995). In addition, as a consequence of the presence of PTCs, decay of the corresponding mRNAs is accelerated resulting in reduced levels of the corresponding transcript (Christiano et al., 1997). Consequently, little if any of the truncated polypeptides are synthesized, thus explaining the negative immunohistochemical staining for type VII collagen epitopes and absence of anchoring fibrils, leading to severe fragility of the skin. Some of these mechanisms are also applicable to the Col7a1 –/– mice which demonstrate extreme skin fragility, as manifested by the formation of large bullae at birth or during the early postnatal period. In fact, these mice die usually within the first two weeks of life unless specially attended. The exact reason for their demise is currently not clear but one explanation would be that the blistering results in profound fluid imbalance. This possibility is attested by the observation that the largest blisters contain fluid sufficient to account for up to 20% of the total body weight of the animals (M. Männikkö and J. F. Klement, unpublished observations). Furthermore, intraperitoneal injections of Ringer's lactate/dextrose solution have been able to extend the life of the affected mice by another 5-10 days (preliminary data). Another explanation for the early demise of the –/– mice may relate to their inability to ingest food due to oral and upper gastrointestinal tract erosions and blisters. In support of this possibility is the observation that one of the –/– mice that lived up to three weeks of age was severely runted. The appearance of this mouse was somewhat similar to recently described desmoglein 3 null mice (Dsg3 –/–) which become runted after 8-10 days of life due to erosive oral lesions inhibiting their food intake (Koch et al., 1997).

The targeted deletion in the Col7a1 gene, replaced by the neomycin resistance gene, spans the exons 46-69. This deletion, which corresponds to the amino-terminal half of the triple-helical collagenous domain of the molecule, would be in-frame, and predicts synthesis of a polypeptide which is 372 amino acids (13%) shorter than the intact proα1(VII) chain. The insertion of the *neo* gene in reverse orientation introduces a stop codon which mimics premature termination codons identified in patients with RDEB (Uitto et al., 1995). As indicated above, PTCs in the type VII collagen gene in patients with RDEB can result in reduced steady-state levels of the corresponding mRNA transcript due to nonsense mediated mRNA decay (Maquat, 1995). In contrast, the expression of Col7a1 mRNA in the skin of –/– mice was detectable at the same level as in +/– or +/+ mice, as

determined by RNase protection assay using a 5′ probe for protection. Thus, the nascent polypeptides translated from the mutant mRNA are either rapidly degraded or unable to assemble into functional type VII collagen molecules. Consequently, the synthesis of type VII collagen polypeptides is abrogated in the −/− mice, resulting in the absence of anchoring fibrils and leading to profound skin fragility and other phenotypic characteristics.

Collectively, our findings indicate that the Col7a1 −/− mice recapitulate many of the clinical, genetic, and ultrastructural features of RDEB. Thus, these mice can serve as an animal model to study the pathomechanisms of DEB in further detail, and provide a system to test various therapeutic approaches, including gene replacement therapies.

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