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Revertant Mosaicism Due to a Second-Site Mutation in *COL7A1* in a Patient with Recessive Dystrophic Epidermolysis Bullosa

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Despite the high incidence of revertant mosaicism (35%) in patients with the genetic skin disease epidermolysis bullosa (EB) due to correcting mutations in the genes *COL17A1* and *LAMB3*, revertant mosaicism has not been described for *COL7A1* until recently. Mutations in *COL7A1* are responsible for the most devastating form of EB in adults, which is characterized by cocooned "mitten" deformities of the hands. This report shows *in vivo* reversion of an inherited *COL7A1* mutation in a patient with recessive dystrophic EB who was homozygous for the frameshift mutation *COL7A1*:c.6527insC,p.2176FsX337. The patient exhibited a patch of clinically healthy revertant skin on her left forearm. The second-site mutation c.6528delT, which is present in revertant keratinocytes, resulted in correction of the reading frame. As the new CCC codon codes for the same amino acid proline as the wild-type codon CCT, the revertant cells expressed wild-type type VII collagen polypeptide, leading to restoration of skin function. We hypothesize that, on careful examination, revertant mosaicism might be found to be more common in patients with type VII collagen-deficient EB. Furthermore, the revertant keratinocytes might offer the possibility to explore cell-based therapeutic strategies, by culturing *in vitro* and subsequently grafting as part of bioengineered dermo-epidermal substitutes on affected skin.

INTRODUCTION

Revertant mosaicism, sometimes also referred to as "natural gene therapy," has been described in several inherited diseases, including Wiskott Aldrich syndrome and Fanconi anemia (Hirschhorn, 2003). It has also been identified in the genetic blistering disease epidermolysis bullosa (EB), which is characterized by skin fragility due to separation of the epidermis from the dermis. Mutations in as many as 13 EB genes can result in diseases in this group. Of the 13 genes, three have been shown to be reverted: *KRT14*, encoding keratin 14 (Schuilenga Hut *et al*., 2002; Smith *et al*., 2004); *LAMB3*, encoding the beta3 chain of laminin 332 (Pasmooij *et al*., 2007); and *COL17A1*, encoding type XVII collagen (Jonkman *et al*., 1997; Darling *et al*., 1999; Pasmooij *et al*., 2005). In addition, a report by Al Aboud *et al*. (2009) suggests an *in vivo* reversion of the *FERMT1* gene in their patient with Kindler's syndrome, as islands of normal skin were present in the affected areas. Incidences of revertant mosaicism of up to 11% have been described in Wiskott Aldrich syndrome (Davis and Candotti, 2009) and up to 18% in Fanconi anemia (Kalb *et al*., 2007). The highest incidence (35%) by any account has been reported for EB, in a subgroup of patients with the nonlethal type of junctional EB due to mutations in *LAMB3* or *COL17A1* (Jonkman and Pasmooij, 2009). Despite the high incidence associated with these two genes, revertant mosaicism had not been described until recently for *COL7A1* (Almaani *et al*., 2010), which encodes type VII collagen, the source of the most devastating form of EB in adults. Here we report on another patient with *in vivo* reversion of an inherited *COL7A1* mutation.

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Abbreviation: EB, epidermolysis bullosa

RESULTS

A 42 year old Spanish woman had a patch of sturdy skin on her left forearm (8 cm × 4.5 cm) where no blisters developed, in contrast to the rest of the affected skin (Figure 1). She was the fourth child of healthy consanguineous parents and the only family member with EB. The generalized congenital blistering with scarring resulted in pseudosyndactyly of the hands starting in infancy. The patient had had hand surgery 2 years before the current presentation. The mucous membranes of the mouth, nose, eyes, and vagina were affected by EB. Hyperpigmentation was evident in areas of the head and neck. Cutaneous squamous cell carcinoma had not developed.

The patch of sturdy skin (revertant) had been noticed by the patient approximately 3 years earlier (Supplementary Video S1 online). Since the initial detection, she had not perceived any remarkable extension. The boundary of the revertant skin was relatively well demarcated. The healthy patch was slightly pigmented, especially at the margin. The hypopigmentation of the surrounding affected skin (mutant) was masked by the erythema, and so the revertant patch stood out as pale. The unblistered skin enabled the patient to wear bracelets on her left arm for the first time in her life. She did not feel any tactile differences between the revertant patch and the affected skin, except for the mechanical resistance. No other areas of clinically revertant skin were identified.

An adhesive strip test with Fixomull tape according to the method of Gostynski *et al*. (2009) confirmed the sturdiness of the revertant skin (Supplementary Video S2 online). In contrast, the adhesive strip test on mutant skin revealed fragile skin. Part of the epidermis was stripped away when the adhesive tape was pulled off (Supplementary Video S3 online).

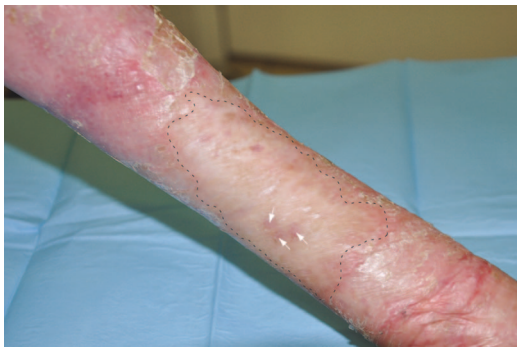

Figure 1. The revertant skin patch was easily distinguishable from the surrounding erythematous atrophic skin. The white arrows point to three small erythematous scars that correspond to the sites of revertant biopsies.

Immunofluorescence microscopy

Two 4 mm fresh frozen skin biopsy samples were obtained: one from the clinically healthy skin of the left forearm (revertant) and the other from the nonlesional affected skin of the right forearm (mutant). The mutant skin showed subepidermal blister formation (Figure 2a). The atrophic epidermis was also noticeably thinner than the revertant skin (Figure 2b) on which no blisters formed. Immunofluorescence staining was performed on both biopsy samples. Type VII collagen staining with all three antibodies tested CALBIO (Calbiochem, Darmstadt, Germany, no. 234192), LH7.2 (Tanaka *et al*., 1994), and 2Q633 (US Biologicals, Swampscott, MA) was almost absent in the mutant skin of the right forearm (1+, Figure 2c) compared with the normal human control skin (4+, Figure 2e). The clinical signs and the very limited type VII collagen staining fit with a diagnosis of severe generalized recessive dystrophic EB (van den Akker *et al*., 2009). Interestingly, immunofluorescence staining for type VII collagen of the revertant skin (Figure 2d) was almost identical in intensity (3+/4+) to that for the control skin (4+) over 85% of the width of the skin biopsy sample. The normal staining of type VII collagen was thus comparable to that of the normal functioning skin of carriers with a single *COL7A1* null mutation. The other 15% showed staining that was comparable to that of the biopsy sample taken from affected skin (1+).

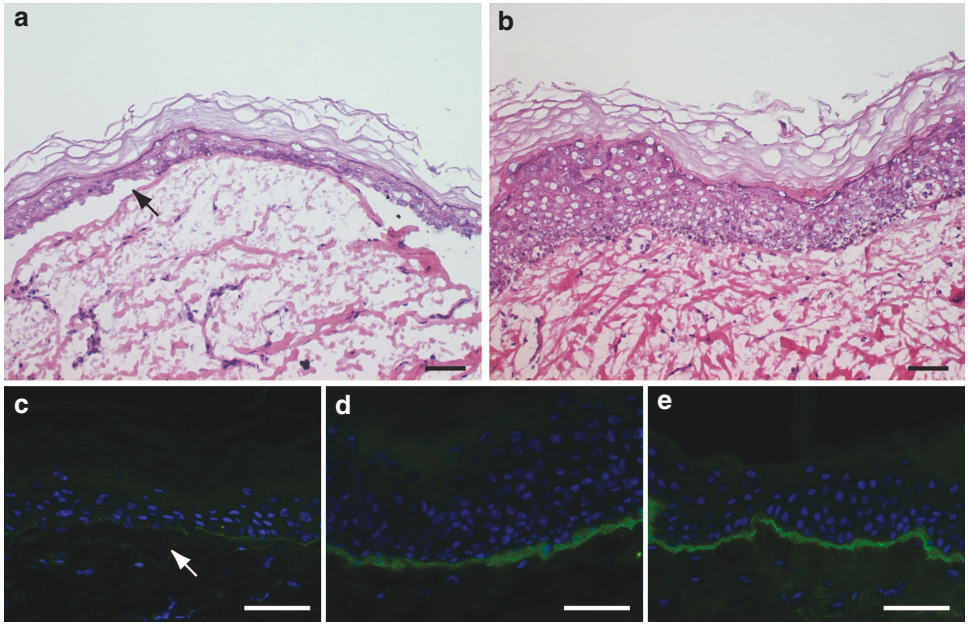

Figure 2. Microscopic analysis of biopsies taken from mutant and revertant skin. The arrows point to subepidermal blister formation in the mutant skin biopsies (**a**, **c**). No blisters were formed in the revertant skin (**b**, **d**). Immunofluorescence staining of type VII collagen with antibody CALBIO was almost absent in the mutant skin of the right forearm (**c**), whereas the revertant skin (**d**) showed staining with CALBIO that was almost the same in intensity as that of the control skin (**e**). Bar = 50 μm.

Identification of inherited *COL7A1* mutations

Subsequently, mutation analysis was performed on DNA isolated from peripheral blood for the *COL7A1* gene, located on chromosome 3p21.31 and coding for type VII collagen, which consists of 2,944 amino acids. The promoter region and all 118 exons and their flanking intronic *COL7A1* sequences were amplified by touchdown PCR (Gardella *et al.*, 1999, 2000). In exon 80, a homozygous insertion of a cytosine nucleotide was identified at position 6,527. This c.6527insC insertion results in a frameshift and a premature termination codon 337 bp downstream of codon 2176 (*COL7A1*:c.6527insC,p.2176FsX337 homozygous). The c.6527insC insertion is a highly recurrent mutation in the Spanish population, accounting for approximately 40% of alleles (Escámez *et al.*, 2010).

Identification of correcting *COL7A1* mutation

To investigate the re expression of type VII collagen in the revertant skin biopsy, laser dissection microscopy was used to separate fibroblasts and revertant keratinocytes with almost normal staining from mutant keratinocytes with reduced staining. Nucleic acids isolated from the dissected specimens were directed to molecular analyses, without the necessity of culturing the keratinocytes *in vitro*. As expected, DNA analyses of the fibroblast samples and the mutant keratinocytes revealed no differences from DNA from peripheral blood surrounding the exon 80 mutation in *COL7A1* (Figure 3a). The c.6527insC insertion was present on both alleles, and no other changes were identified. Very interestingly, the DNA isolated from the revertant keratinocytes revealed an additional mutation one nucleotide downstream of the inherited c.6527insC mutation. This somatic c.6528delT mutation leads to correction of the disturbed reading frame. The new CCC codon codes for the same amino acid proline as the wild type codon CCT, thus resulting in wild type type VII collagen polypeptide (Figure 3b).

RNA analysis performed on RNA isolated from the revertant skin biopsy specimen was in line with the DNA findings. RNA isolated from fibroblasts and keratinocytes with low type VII collagen expression did not contain the somatic second site reversion mutation.

DISCUSSION

Our 42 year old female patient is, to our knowledge, the second recessive dystrophic EB case described with documented revertant mosaicism. Type VII collagen is formed by both keratinocytes and fibroblasts. Gene therapy experiments have shown that correction of the *COL7A1* gene in either keratinocytes or fibroblasts can result in re expression of the type VII collagen protein and restoration of skin function (Goto *et al.*, 2006). In the revertant skin of our patient, the keratinocytes with normal type VII collagen expression were genetically corrected, whereas the fibroblasts were unchanged. This observation is in line with what has been reported previously for *in vivo* reversion of *COL17A1*, *LAMB3*, and *KRT14*, in which the fibroblasts were also unchanged.

Different reversion mechanisms, such as gene conversion or an additional second site mutation, might underlie the

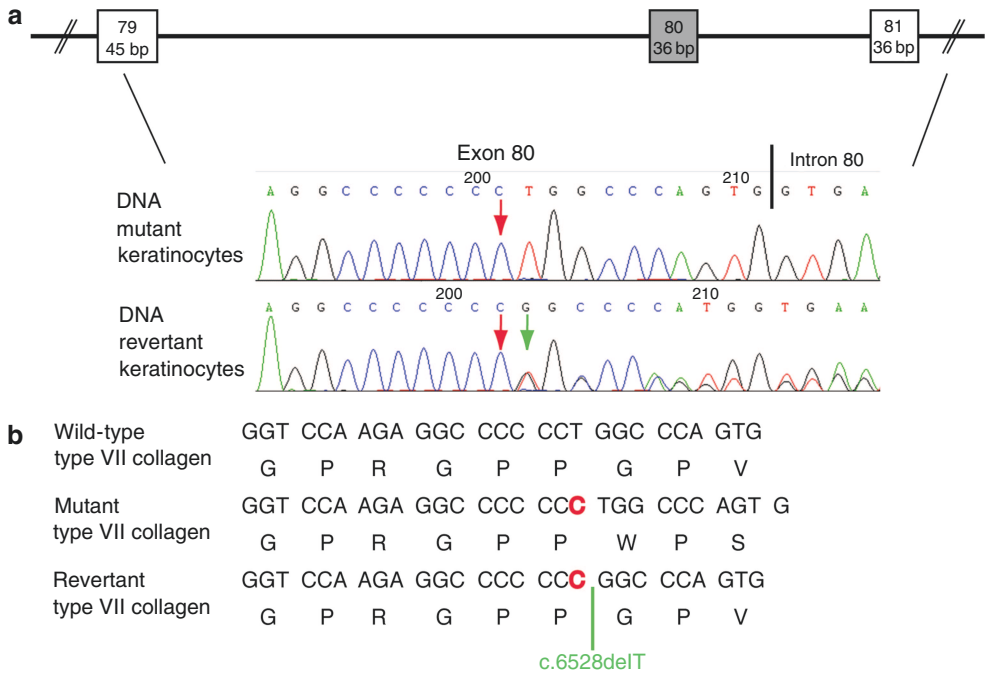

Figure 3. DNA analysis of mutant and revertant keratinocytes. (**a**) The c.6527insC mutation is present in mutant keratinocytes with reduced staining for type VII collagen and also in revertant keratinocytes with almost normal staining for type VII collagen (red arrow). In addition, the revertant keratinocytes carry the second-site mutation c.6528delT mutation on one allele. (**b**) The amino acid sequences deduced from wild-type *COL7A1*, *COL7A1* with c.6527insC (mutant keratinocytes), and *COL7A1* with c.6527insC and c.6528delT (revertant keratinocytes), respectively.

in vivo reversion of a frameshift mutation. For instance, a secondary mutation has been reported for the c.4003delCT *COL17A1* mutation, in which the additional frameshift c.4080insGG restored the reading frame (Darling *et al.*, 1999), as well as for the c.4424 5insC *COL17A1* mutation, in which the additional splice site c.4463 1G>A mutation resulted in skipping of the mutated exon and in formation of a functional protein (Pasmooij *et al.*, 2005). In contrast to these two cases in which a slightly different protein was formed, the secondary c.6528delT mutation in our case is responsible for restoration of type VII collagen protein expression with the wild type amino acid sequence.

Like a previously reported patient with EB due to the *LAMB3* mutations (c.628G>A, c.1903C>T) (Pasmooij *et al.*, 2007), our patient claimed that her revertant skin had developed after birth. She had noticed it approximately 3 years before the current presentation. The clinical history therefore suggests postnatal cell expansion of a revertant cell. In contrast, in patients with *in vivo* reversion of *COL17A1* encoding type XVII collagen, the revertant skin patches had been present as long as the patients could remember and they had not changed in size, even under wound healing conditions (Pasmooij *et al.*, 2005). Our patient will be closely followed for changes in the size of the skin patch on the forearm, as well as for other patches that might develop.

It is not yet clear at what time point the c.6528delT mutation took place. The *in vivo* genomic correction event might have occurred several years earlier, immediately followed by expansion of the revertant cell, or it could be that the event occurred during embryogenesis in a cell that began to expand later in life. If the first scenario is true, the question that arises is why these correcting mutations occur in keratinocytes and not in fibroblasts. Moreover, in the other reported patient with revertant mosaicism in recessive dystrophic EB, only the keratinocytes were genetically corrected (Almaani *et al.*, 2010). One might hypothesize that, given that the epidermis is continually exposed to environmental insults and in a constant state of renewal, the epidermal stem cells have a greater chance of incorporating mutations. In support of this hypothesis is the finding that tissues that normally undergo rapid renewal, such as the skin, lungs, and gastrointestinal tract, have a higher probability of an oncogenic event. It is unlikely, however, that UV exposure has had a role in the occurrence of the correcting c.6528delT mutation. G:C→A:T transitions are considered to be UVB induced when occurring at tandem dipyrimidine sites or pyrimidine runs (Brash *et al.*, 1991), whereas the most common transition induced by UVA is a A:T→C:G transversion (Drobetsky *et al.*, 1995). Given that the c.6528delT mutation is neither a transition nor a transversion, the role of UV is negligible.

The revertant keratinocytes might offer therapeutic possibilities for culturing these clinically healthy keratinocytes *in vitro* and subsequently grafting on affected skin of patients with one of the severe types of EB. Although functional repair was not achieved in one patient with junctional EB owing to the low percentage of revertant cells in the graft (Gostynski *et al.*, 2009), the adhesive stripping procedure was proved to be effective. Further research into revertant cell therapy is required before it can be brought into clinical practice.

As with *COL17A1*, in which revertant mosaicism was also believed to be an extremely rare event, we speculate that, on careful examination, revertant mosaicism might be found to be more common in patients with type VII collagen deficient dystrophic EB.

MATERIALS AND METHODS

Informed consent

Informed consent was obtained from the patient in accordance with the policies of the collaborative centers where biopsy samples and blood DNA samples were obtained. The Ethics Committee of Hospital Sant Joan de Reus evaluated and approved this research work, stating that the project adheres to the Helsinki Guidelines and further reviews (Edinburgh, 2000; http://www.wma.net/en/30publications/30ethicsmanual/pdf/ethics manual en.pdf).

Adhesive strip test

The adhesive strip test was performed according to the method described by Gostynski *et al.* (2009). Two strips (each at least 1 cm × 2 cm) of Fixomull Stretch adhesive tape (Beiersdorf, Hamburg, Germany) were cut. After the skin was degreased with ether, the adhesive tape was firmly affixed to the selected skin. After 24 hours, the strips were fiercely pulled off with forceps.

Immunofluorescence microscopy

For detection of type VII collagen, three antibodies were used: mouse monoclonal antibody LH7.2, monoclonal mouse antibody 2Q633, and polyclonal rabbit antibody CALBIO.

Mutation detection

Genomic DNA was extracted from blood using the QIAamp DNA Blood Maxi Kit (Qiagen, Venlo, The Netherlands). The promoter region and all 118 exons and their flanking intronic *COL7A1* (Genbank: NM000094) sequences were amplified by touchdown PCR (Gardella *et al.*, 1999, 2000).

DNA isolation of laser dissection microscopy samples

For DNA recovery, 4 μm skin cryosections were stained with antibody LH7.2. Cells were dissected using the Leica (Leica Microsystems Nussloch GmbH, Nussloch, Germany) Laser Microdissection system 6000. Dissected areas were collected in a 0.2 ml tube with a flat cap, containing 25 μl of one time PCR buffer (Amersham Pharmacia Biotech, Uppsala, Sweden) and 1 μl of proteinase K (Invitrogen, Paisley, UK). During digestion by proteinase K, the tubes were kept inverted for 60 minutes at 55 °C; subsequent heating to 98 °C for 15 minutes inactivated the proteinase K. The final aliquots were used for PCR.

RNA isolation

For RNA isolation, four 10 μm skin sections were lysed in 100 μl of lysis buffer plus 0.7 μl of β mercaptoethanol. Total RNA (15 μl) was prepared using the Stratagene RNA microprep kit (Stratagene).

cDNA synthesis

For cDNA synthesis, 1 μl of 300 ng per μl of random primers (Invitrogen) and 1 μl of 10 mM dNTP mix (Fermentas, St Leon Rot,

Germany) were added to 10 µl of total RNA and incubated for 5 minutes at 65 °C. Subsequently, 4 µl of 5 × First Strand buffer, 2 µl of 0.1 M dithiothreitol, 1 µl of 10 units per µl of RNAse Inhibitor Cloned, and 1 µl of 1 unit per µl of Superscript II Rnase H^- Reverse Transcriptase (all from Invitrogen) were added. Tubes were incubated for 5 minutes at room temperature and for 60 minutes at 50 °C, followed by 15 minutes at 70 °C to inactivate the enzyme.

Identification of mutations in skin samples

For detection of mutations in laser dissection microscopy isolated DNA, we used nested PCR. Primer sequences are listed in Supplementary Table S1 online. The cDNA samples from skin sections were also subjected to nested PCR. The primer sequences are listed in Supplementary Table S2 online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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