Natural Gene Therapy May Occur in All Patients with Generalized Non-Herlitz Junctional Epidermolysis Bullosa with *COL17A1* Mutations

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Mutations in the type XVII collagen gene (*COL17A1*) result in the blistering disorder non-Herlitz junctional epidermolysis bullosa (JEB-nH). The incidence of revertant mosaicism, also called "natural gene therapy", was identified in a cohort of 14 patients with JEB-nH caused by *COL17A1* mutations in the Netherlands. Five different *in vivo* reversions, all correcting the germ-line *COL17A1* mutation c.2237delG in exon 30, were found in four mosaic JEB-nH patients. The correcting DNA changes involved a wide variety of somatic mutations, from which an indel mutation (c.2228-101_2263+70delins15) and a large deletion of 2,165 base pairs (c.2227+153_2336-318del) have not been previously observed in patients with revertant mosaicism. Our results show that there is no preference for a repair mechanism. Moreover, revertant mosaicism was confirmed on a DNA level in 6 out of 10 generalized JEB-nH patients. Further, photo-material and clinical history of the other four generalized JEB-nH patients demonstrated that each patient has revertant skin patches. In contrast, revertant mosaicism was not detected in the four localized JEB-nH patients. The fact that so many, if not all, generalized JEB-nH *COL17A1* patients have revertant patches offers opportunities for cell therapies in which the patient's own naturally corrected cells are used as a source.

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INTRODUCTION

Revertant mosaicism refers to the coexistence of cells carrying inherited genetic mutations with cells in which the inherited disease-causing mutation is corrected by a spontaneous genetic event, thereby giving rise to a clinically healthy phenotype. This phenomenon has been reported in several genetic disorders, affecting self-regenerating organ systems, such as skin, blood, and liver (for review see Jonkman, 1999; Hirschhorn, 2003; Lai-Cheong *et al.*, 2011a). Several genetic mechanisms can underlie this natural gene therapy, including true back mutation, intragenic crossover, mitotic gene conversion, and second-site mutation (Kvittingen *et al.*, 1994; Ellis *et al.*, 1995; Jonkman *et al.*, 1997).

In skin, *in vivo* reversion has been described for the genetic blistering disorder epidermolysis bullosa (EB), which is characterized by skin fragility that is accompanied by blister formation. Mutations in as many as 15 different EB genes can result in this group of genetic skin disorders. Of these 15 genes, five have shown to be reverted, i.e., *KRT14* encoding keratin 14 (Schuilenga-Hut *et al.*, 2002; Smith *et al.*, 2004), *LAMB3* encoding the β3 chain of laminin-332 (Pasmooij *et al.*, 2007a), *COL17A1* encoding type XVII collagen (Col17, Jonkman *et al.*, 1997; Darling *et al.*, 1999; Pasmooij *et al.*, 2005), *COL7A1* encoding type VII collagen (Almaani *et al.*, 2010; Pasmooij *et al.*, 2010; Van den Akker *et al.*, 2011), and *FERMT1* encoding kindlin-1 (Lai-Cheong *et al.*, 2011b).

Initially, revertant mosaicism was thought to be a rare phenomenon. Recently, however, we showed that 35% of the patients with non-Herlitz junctional EB (JEB-nH), due to mutations in *COL17A1* or *LAMB3,* carry revertant patches, indicating that revertant mosaicism is more common than was previously anticipated (Jonkman and Pasmooij, 2009). These clinically healthy skin areas manifested as patches of homogeneously pigmented skin in the patients with *COL17A1* mutations. Another amazing example of the frequency of these *in vivo* reversions is the presence of hundreds or thousands of pale confetti-like revertant spots that appear across the body surface in the patients with ichthyosis with confetti, because of dominant *KRT10* mutations that change the reading frame and produce an arginine-rich C-terminal peptide (Choate *et al.*, 2010). Each reversion was because of mitotic recombination. Unlike these patients, we report here on four patients with *COL17A1* mutations in

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Abbreviations: BMZ, basement membrane zone; bp, base pairs; Col17, type XVII collagen; EB, epidermolysis bullosa; EM, electron microscopy; IF, immunofluorescence microscopy; iPS, induced pluripotent stem; JEB-nH, non-Herlitz junctional epidermolysis bullosa

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which the inherited c.2237delG mutation was corrected in five different ways, showing that there is no preference for a certain correction mechanism. Moreover, we investigated the incidence of natural gene therapy in JEB-nH patients in the Netherlands, indicating that the majority, if not all, of the JEB-nH patients carry one or more revertant patches.

RESULTS

Four patients with type XVII collagen–deficient generalized JEB-nH were studied. Patients EB035-01 and EB035-02 were brothers and both were compound heterozygous for a frameshift mutation in *COL17A1* (human *COL17A1* messenger RNA (mRNA) sequence: GenBank accession number NM_000494.3) in exon 30, c.2237delG;p.Gly746AlafsX53, and a nonsense mutation in exon 51, c.3676C>T;p.Arg1226X (Pasmooij *et al.*, 2007b). These mutations were previously referred to as c.2342delG and c.3781C>T, respectively. This difference in numbering occurs because the initial nomenclature by Giudice *et al.* (1992) for the *COL17A1* gene included 105 additional nucleotides before the start codon was reached, whereas the current nomenclature starts with number 1 at the site of the start codon. The third (EB208-01) and fourth (EB025-01) patients were homozygous for c.2237delG, which is a common founder mutation in the Netherlands (Scheffer *et al.*, 1997).

Patient EB035-01

Patients EB035-01 and EB035-02 were born to nonconsanguineous parents. They have two brothers without a blistering disease. Patient EB035-01, a 48-year-old man, had blisters and large erosions since birth. Upon clinical examination, we noted normal skin on the knees with subpattelar calluses (Figure 1d). The patient indicated that he walked on his knees indoors, because that skin area developed no blisters. The dorsum of the hands showed large hyperpigmented macules standing out against the pale, hypopigmented, surrounding skin (Figure 2a and b).

Three biopsies R1, R2, and R3 were taken from hyperpigmented clinically healthy skin where no blisters occurred, and one biopsy M1 was taken from the pale affected skin for immunofluorescence microscopy (IF) and electron microscopy (EM; Figure 1d). Staining for Col17 with NCC-Lu-226 in the affected skin biopsy (Figure 1b) was almost completely absent along the basement membrane zone (BMZ) compared with normal human control skin (4+, Figure 1a). In contrast, all biopsies taken from revertant skin showed reexpression of Col17 (Figure 1a) with NCC-Lu-226 staining, corresponding to about 75% of the intensity of normal human control skin (Figure 1e, f, and g). Staining for 1D1 against the C-terminal part of the ectodomain of Col17 was absent in the affected skin biopsy, whereas reduced expression and loss of lateral staining was observed in the revertant keratinocytes, corresponding with the staining pattern of heterozygous carriers of a recessive *COL17A1* mutation (Pasmooij *et al.*, 2007b). The fraction of keratinocytes that reexpressed Col17 along the BMZ varied from 8/10 in biopsy R1 to 9/10 in biopsies R2 and R3. A tape strip test in which a small piece of tape was placed on the skin and afterwards removed with tweezers (Gostynski *et al.*, 2009) showed no blister formation in the revertant skin (Figure 1c), whereas peeling the epidermis in affected skin resulted in epidermal–dermal separation (data not shown).

EM showed hemidesmosomes in revertant skin (R1 and R3, data not shown) that were normal in shape and number. In the biopsy taken from the left knee (R2), some basal cells had normal hemidesmosomes, whereas other basal cells had a low number of hypoplastic hemidesmosomes, which fits with mosaicism at a cellular level. In line with the JEB-nH phenotype, affected skin (M1) revealed a split through the lamina lucida.

Patient EB035-02

Patient EB035-02, brother of patient EB035-01, who is 1.5 years younger, also presented with blisters after minor trauma since birth. Clearly defined hyperpigmented areas were present on the face, forehead, and dorsal sides of the hands (Figure 2c). Similar to his brother, the patient did not know whether the clinically unaffected patches were present already at birth or had developed later in life.

Immunofluorescence staining for Col17 of the biopsy (M2) from pale affected skin was absent with antibodies 1D1 and 1A8C, and almost absent with antibodies 233 and NCC-Lu-226. Interestingly, the biopsy of the hyperpigmented patch of the hand (R4) showed reexpression of Col17 over approximately 4/5 of the BMZ, whereas the other 1/5 resembled the staining of the mutant skin biopsy (data not shown).

EM analysis of mutant skin revealed a lucidolytic split with the basal membrane on the blister floor. No blister formation was observed in the revertant skin (R4). Although the hemidesmosomes were partly hypoplastic, the basal cells had correct tonofilament projections to the hemidesmosomes.

Patient EB208-01

Patient EB208-01 was seen for the first time in our clinic at the age of 46 years. He was the sixth child of nonconsanguineous parents. One brother and one sister had a blistering disease and had died during the first year of their lives. In addition, his mother had generalized blistering with alopecia. No other family members were affected. No DNA of any of the family members was available. Physical examination showed generalized blistering, especially on the lower arms and palms. Erosions were present on the left upper leg, right ankle, and lower back. The patient had atrophic alopecia of frontal scalp, eyebrows, eyelashes, and beard, and had no secondary hair. Two hyperpigmented skin patches on the dorsal side of the wrist did not show blisters after rubbing (Figure 2d and e). The patient indicated that he had noticed the first patch at around 10 years of age (Figure 2d). The other non-blistering skin patch appeared later in life (Figure 2e). Pictures taken in 2008 and 2011 revealed that the hyperpigmented patch did not change in size.

IF antigen mapping for Col17 (1D1) of pale affected skin (M3) was negative (Pasmooij *et al.*, 2007b). Instead, the biopsies of hyperpigmented revertant skin were positive for 1D1 in about 6/10 and 9/10 of the basal cells in biopsies R5

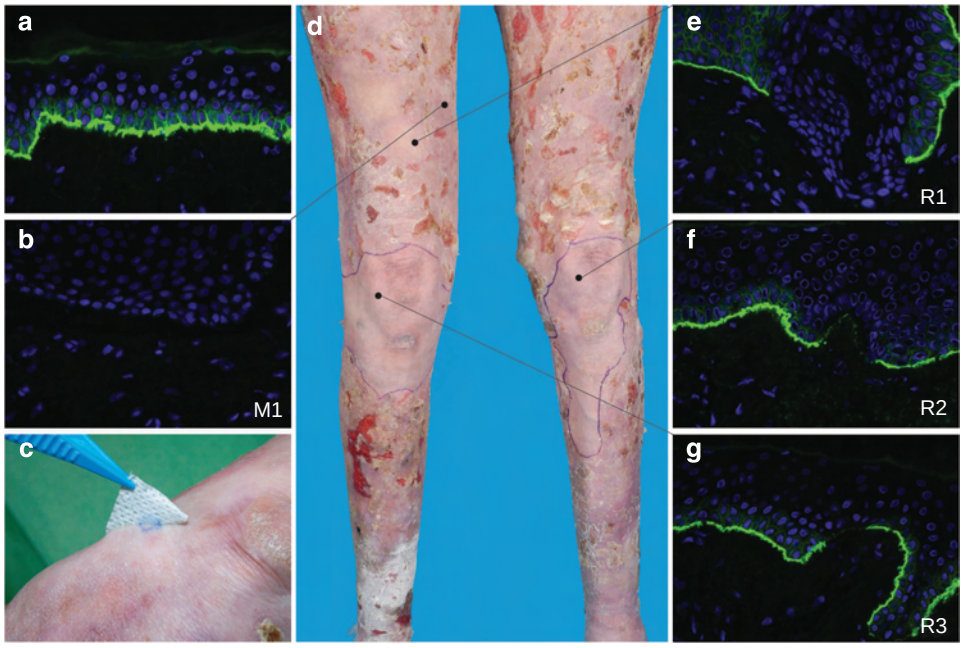

Figure 1. Revertant biopsies of patient EB035-01. Absence of blister formation was noted on both the knees. (**d**) In addition, at least two round patches of clinically healthy skin were present above the right knee. (**b**) A biopsy taken from affected skin showed lack of Col17 expression (M1) compared with (**a**) normal human control skin. (**e**, **f**, **g**) Instead, all biopsies taken from unaffected skin (R1, R2, R3) showed reexpression of Col17. (**c**) A tape strip test showed absence of blister formation in the revertant skin.

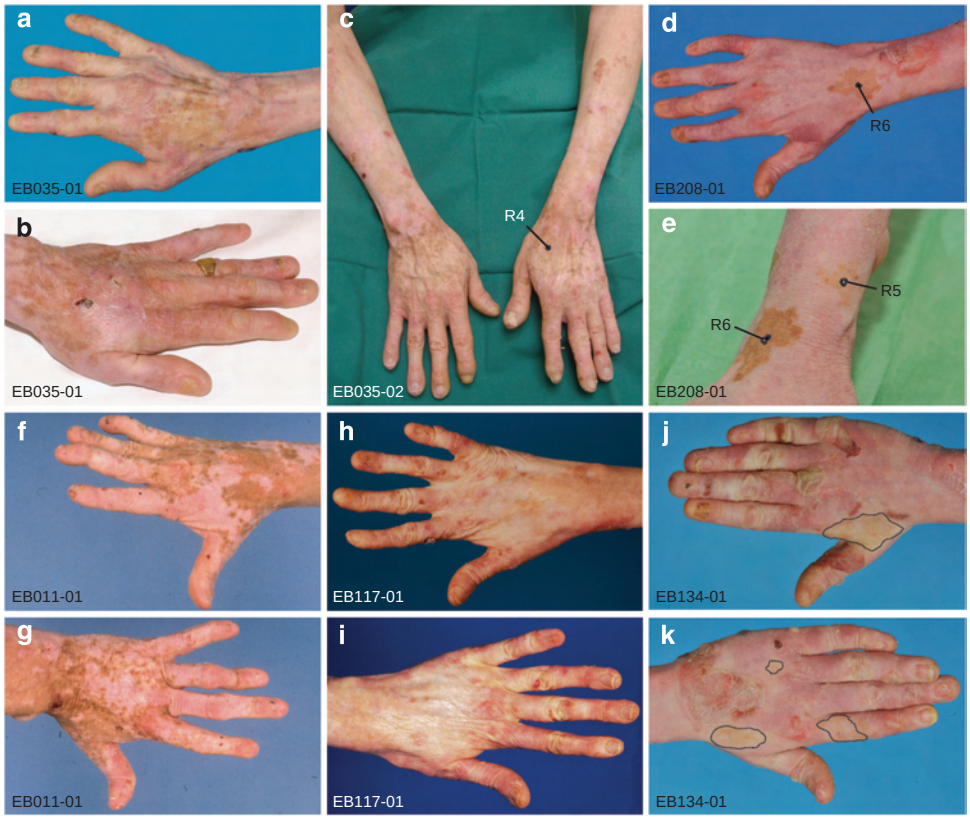

Figure 2. Revertant skin areas in generalized JEB-nH patients. (**a**, **b**) The pigmented patches of patient EB035-01 on both his hands resembled the pigmented patches of his brother, i.e., (**c**) patient EB035-02. In a skin biopsy of patient EB035-02, revertant mosaicism was confirmed (R4). (**d**, **e**) The right hand of patient EB208-01 showed two pigmented macular patches (R6 and R5, respectively). The positions of the biopsies are marked with black circles. (**f**, **g**) The hands of patients EB011-01 and (**h**, **i**) EB117-001 also show similar pigmented areas on both the hands. (**j**, **k**) In the 8-year-old patient EB134-01, the pigmented, non-blistering areas are indicated. Clearly visible are the blisters that occur in the surrounding area.

and R6, respectively. The 1D1 staining of the revertant keratinocytes was slightly reduced compared with normal human control skin and the apical-lateral staining was lacking.

EM showed hypoplastic hemidesmosomes with absence of the subbasal dense plate in affected skin of the upper leg (Supplementary Figure S1a online). The number of hemidesmosomes was reduced. The revertant skin biopsy (R6) showed basal cells with hypoplastic hemidesmosomes and basal cells with normal-sized hemidesmosomes (Supplementary Figure S1b online), fitting with cellular mosaicism. Most of the hemidesmosomes had a subbasal dense plate. No EM biopsy had been taken of the other revertant skin area.

Patient EB025-01

In 1994, patient EB025-01 last visited our polyclinic. Fifteen years later, upon clinical examination, hyperpigmented areas were noted on her back (Figure 3a), both the hands, and lower and upper arms (Figures 3b and c). The patient indicated that she did not develop blisters in these areas, although she was unsure whether the clinically healthy areas had expanded or how long they had been present. Although she kindly provided us with pictures from her childhood, the resolution was unfortunately too low to be helpful in determining when these healthy patches had developed. Interesting was the presence of some hairs in the unaffected skin area on her arms (Figure 3d), whereas in the affected patches hairs were absent. Staining with 233 showed absence of Col17 in the affected skin biopsy (M4), whereas 3/4 of the basal cells in the unaffected skin biopsy (R7) expressed Col17 protein to an almost normal extent as in normal control skin.

DNA analysis of revertant skin biopsies

To investigate the correction mechanism, laser dissection microscopy was used to separate revertant keratinocytes from mutant keratinocytes and from fibroblasts. Subsequent DNA analysis of Col17-positive keratinocytes of biopsies from distinct revertant patches showed different second-site mutations (Supplementary Figure S2 online): c.2228-101_2263+70delinsCTCTTAACCATAGTG in biopsy R1, c.2259_2263+9del in biopsy R2, c.2227+153_2336-318del in biopsy R5, c.2238C>T in biopsy R6, and c.2263+2T>C in biopsies R3, R4, and R7. The c.2238C>T substitution in combination with the germ-line c.2237delG mutation changes the amino acid on position 746 from glycine (GGC) to valine (GTC). None of the second-site mutations was detected in DNA of mutant keratinocytes or in fibroblasts.

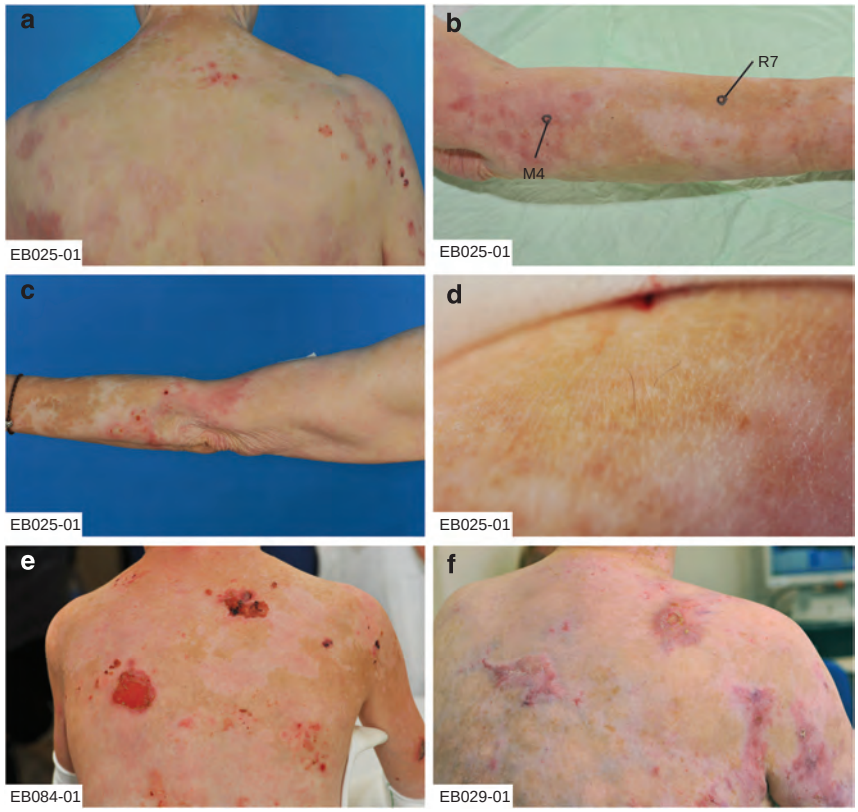

Figure 3. Revertant skin in patients EB025-01, EB084-01, and EB029-01. Several clinically unaffected areas were present on the (**a**) back, and (**b**) lower and (**c**) upper arms. After sun exposure, the clinically unaffected areas become easily noticeable because of their hyperpigmentation compared with the surrounded skin that is hypopigmented. Note this difference in (**a**) the neck of the patient and (**b**) on the lower arm. (**d**) Occasionally, a hair was present in the healthy skin area. (**e**) On the back of the 11-year-old boy, EB084-01, hyperpigmented areas were noticed that resembled the revertant skin patches of patient EB025-01 for which *in vivo* reversion was confirmed on a protein and DNA level. (**f**) In patient EB029-01 with JEB-nH due to laminin-332 deficiency (Pasmooij *et al.*, 2007a), the revertant skin areas were also pigmented compared with the surrounding affected skin.

The indel mutation c.2228-101_2263+70delins15 in R2 deleted 206 base pairs (bp), including the last 101 bp of intron 29, complete exon 30, and the first 70 bp of intron 30. Instead, 15 bp were inserted, of which 13 of the 15 bp corresponded with a sequence ''CTTAACCATAGTG'' that was present 3 bp after the 206-bp deletion. The large deletion in R5, c.2227+153_2336-318del, consisted of 2,165 bp and resulted in absence of exons 30 and 31. The indel mutation c.2228-101_2263+70delins15 and large deletion c.2227+153_2336-318del are therefore predicted to result in mRNA with skipping of exon 30, and skipping of exons 30 and 31, respectively.

The consequence of the somatic c.2259_2263+9del, c.2263+2T>C, and c.2238C>T mutations was checked with the splice-site prediction program NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/). Both c.2259_2263+9del and c.2263+2T>C abolished the 5′-donor splice site of intron 30, hence skipping of exon 30 in the mRNA would be expected. In contrast, the c.2238C>T transition was not predicted to have a significant effect on either the 3′-acceptor splice site of intron 29 or on the 5′-donor splice site of intron 30.

RNA analysis of revertant skin biopsies

Four of the five second-site mutations, c.2228-101_2263+70delins15, c.2259_2263+9del, c.2263+2T>C, and c.2238C>T, affected splicing in a similar manner, by giving rise to an exon 30–deleted in-frame mRNA transcript (Figure 4). As expected, mRNA analysis of the revertant keratinocytes with the large deletion c.2227+153_2336-318del showed an in-frame transcript with a 108-bp deletion of exons 30 and 31.

DISCUSSION

This study describes in four patients the *in vivo* reversion of the c.2237delG *COL17A1* mutation due to five different second-site changes in seven skin biopsy specimens. The correcting DNA changes involved a wide variety of different mutations, i.e., a transition in the same codon (c.2238C>T), a substitution in the 5′-donor splice site of intron 30 (c.2263+2T>C), a deletion in exon 30 and intron 30 (c.2259_2263+9del), an indel mutation (c.2228-101_2263+70delins15), and a 2,165-bp deletion (c.2227+153_2336-318del). Although many second-site mutations have been discovered in patients with revertant mosaicism, an indel mutation and a deletion of more than 2,000 bp that revert the phenotype to normal have not been previously observed.

Four of the five *COL17A1* second-site mutations had the same effect on the mRNA level, i.e., exon 30 was spliced out, which resulted in a Col17 protein with a deletion of 12 amino acids. This slightly smaller Col17 protein is functional, as could be observed by the adhesive strip test that was performed on revertant skin of the knee in patient EB035-01. In addition, the mild localized phenotype of a previously published JEB-nH patient EB098-01 (c.2251C>T;p.Gln751X, c.3327delT) with outsplicing of the premature termination codon–containing exon 30 illustrates the functionality of the exon 30–deleted Col17 protein (Pasmooij *et al.*, 2004b). The second-site mutation c.2238C>T changed the amino acid at position 746 to a valine. Although the program NetGene2 predicted no significant effect on mRNA splicing, exon 30 was skipped out. Subsequent analysis of exonic splicing enhancers with ESEfinder (Cartegni *et al.*, 2003) showed that the combination of the c.2237delG and c.2238C>T mutation compared with the wild-type sequence resulted in the abolishment of two exonic splicing enhancers, and a minor increase in score from a SF2/ASF site from 1.94 to 2.14. It might be that the outsplicing of exon 30 is the result of this increase in the SF2/ASF site. Another factor that could have influenced splice-site selection is a possible effect of the c.2238C>T on the RNA secondary structure, which in turn may affect splicing (Shen *et al.*, 1999; Buratti and Baralle, 2004). The mRNA data of the second-site mutation in R5 were aberrant from the other revertant biopsies, as deletion of exons 30 and 31 was observed. This mRNA transcript resulted in a functional Col17 protein with a deletion of 36 amino acids in the largest collagenous domain (Col15).

The clinically healthy skin patches all originated from a distinct *in vivo* reversion event, because the correcting *COL17A1* mutations were all different within one patient. Moreover, our results show that there is not a single preferred mechanism for correction of the inherited c.2237delG *COL17A1* mutation. Similarly, in Wiskott–Aldrich Syndrome multiple revertant clones were observed within one patient correcting the same germ-line mutation. Davis *et al.* (2010) described a patient with at least 38 different revertant genotypes, comprising of deletions and base substitutions that restored expression of WASp. In contrast, in patients with ''ichthyosis with confetti'' due to dominant *KRT10* mutations, the repair mechanism was in all normal skin spots mitotic recombination of variable length (Choate *et al.*, 2010). The authors speculated that the mislocalization of the mutant keratin 10 to the nucleolus might have elevated the mutation rate for mitotic recombination, thereby resulting in thousands of revertant clones.

Depending on the EB gene and the nature and location of the inherited mutation, a certain germ-line mutation will have more possibilities for correction than another inherited mutation. It is known that skipping of exons 30 or 33 of *COL17A1* both lead to a milder phenotype (Ruzzi *et al.*, 2001; Pasmooij *et al.*, 2004b). The c.2237delG *COL17A1* mutation can thus be repaired by all mutations that result in deletion of exon 30, whereas it is unknown whether deletion of the 147-bp-containing exon 51 can result in a functional Col17 protein. The c.3676C>T mutation in exon 51 was not repaired in patients EB035-01 and EB035-02. Nonetheless, correction of this mutation has occurred in other patients, e.g., by a second-site mutation in the same codon (c.3677G>C) changing the nonsense to a missense codon (Pasmooij *et al.*, 2005).

Natural gene therapy has now been confirmed on a DNA level in the majority of the generalized JEB-nH patients with *COL17A1* mutations in the Netherlands, i.e., 6 out of 10 (60%), when the patients described in this article are included. In fact, studying the photo-material of the other four JEB-nH *COL17A1* patients with generalized blistering

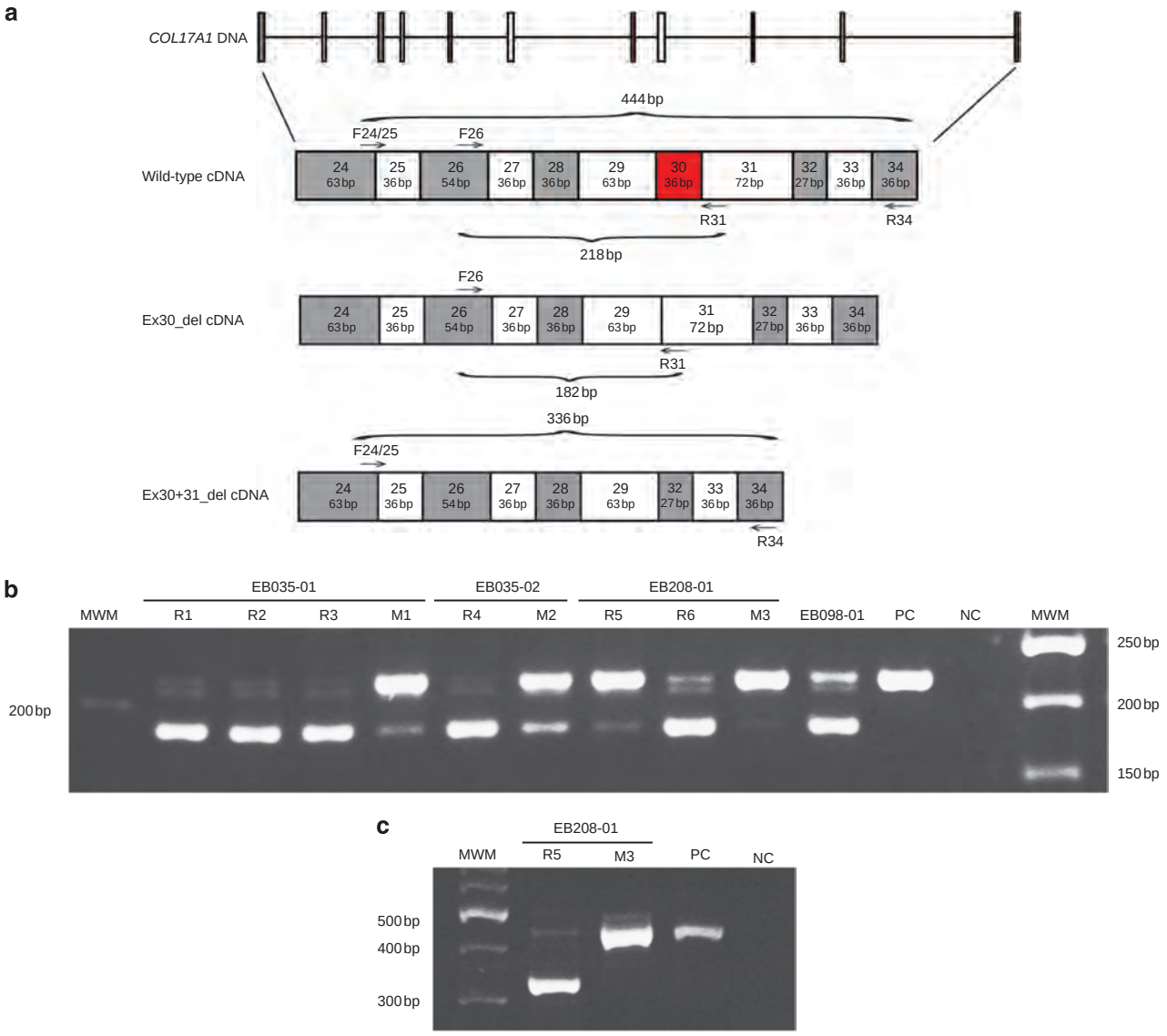

Figure 4. *COL17A1* messenger RNA (mRNA) analysis of revertant and mutant skin biopsies. (**a**) Schematic depiction of the *COL17A1* gene with introns and exons (boxes) with different complementary DNA (cDNA) transcripts of wild-type, Ex30_del, and Ex30 + 31_del given below. (**b**) Agarose gel of cDNA amplified with primers F26 and R31. Lane EB098-01 represents cDNA from a patient who is known to have exon 30-skipping (Pasmooij *et al.*, 2004b). Wild-type cDNA resulted in a transcript of 218 bp, whereas the exon 30–deleted transcript was 182 bp in size. For the revertant biopsies R1, R2, R3, R4, and R6, almost all cDNA originated from the corrected *COL17A1* allele. For revertant biopsy R5 amplified with primers F26 and R31, only cDNA was detected from the mutant *COL17A1* allele carrying the c.2237delG mutation. (**c**) Agarose gel of cDNA amplified with primers F24/25 and R34. Wild-type cDNA resulted in a transcript of 444 bp. When cDNA of biopsy R5 was amplified with primers F24/25 and R34, a transcript of 336 bp was observed that lacked both exons 30 and 31 that originated from the corrected *COL17A1* allele with the large deletion c.2227 + 153_2336-318del. This transcript lacking exons 30 and 31 could not be amplified with the primers F26 and R31 as used in agarose gel (**b**), as the sequence to which R31 attaches was missing in the shortened cDNA transcript. MWM, molecular weight marker; NC, negative control; PC, positive control.

(patients published in Jonkman and Pasmooij (2009)) indicates in each patient one or more pigmented areas without blister formation that are highly suspected for natural gene therapy (Figures 2h, i, j, k, and 3e), as the areas closely resemble the revertant skin patches of the patients for whom *in vivo* reversion has been confirmed on a protein and DNA level. No skin biopsies were taken from these four patients, of whom two patients are deceased (EB011-01 and EB117-01) and two patients are children (EB084-01 and EB134-01). EB nevi can also be hyperpigmented, although EB nevi can be distinguished from the revertant skin patches, as EB nevi are often irregularly pigmented plaques, which may give rise to small satellite nevi surrounding the primary nevus (Lanschuetzer *et al.*, 2010), whereas the revertant areas are homogenously pigmented macules without satellites. As the pigmented clinically healthy patches are very typical for revertant mosaicism in these *COL17A1* patients, we believe it is fair to conclude that the majority, if not all patients with a generalized nH-JEB phenotype have revertant mosaicism (Table 1). In addition, in patients with JEB-nH due to *LAMB3*

Table 1. Patients with generalized JEB-nH due to *COL17A1* mutations in the Netherlands

Patient number	Current age (years)	Revertant area confirmed by DNA	Other revertant areas	Allele 1	Molecular reversion event	Consequence	Allele 2	Molecular reversion event	Consequence
011-01	43[2]		Both hands and legs	c.2237delG			c.2237delG		
025-01[1]	59	Right lower arm	Hands, left lower arm, both upper arms, and back	c.2237delG	c.2263+2T>C	Exon skipping of 36-bp exon 30	c.2237delG		
026-01	44	Both lower arms, hands, and left ankle	Right lower leg, right ankle, left upper arm, back and scalp	c.1601delA	Gene conversion 1 Gene conversion 2 Gene conversion 3	Wild-type Col17 Wild-type Col17 Wild-type Col17	c3676C>T; p.Arg1226X	c.3677G>C	Col17 protein with one different amino acid (p.Arg1226Ser).
035-01	49[2]	Both knees, several patches on right upper leg	Both hands	c.2237delG	c.2228-101_2263+70delins15 c.2259_2263+9del c.2263+2T>C	Exon skipping of 36-bp exon 30 Exon skipping of 36-bp exon 30 Exon skipping of 36-bp exon 30	c.3676C>T; p.Arg1226X		
035-02	51	Left hand	Right hand, both lower arms, left upper arm, forehead, and face	c.2237delG	c.2263+2T>C	Exon skipping of 36-bp exon 30	c.3676C>T; p.Arg1226X		
084-01	11		Both hands, left lower arm, and several patches on back	c.1179delA			c.3327delT		
093-01	75[2]	Right middle finger	Both hands and scalp	c.3676C>T; p.Arg1226X	c.3676T>C or gene conversion	Wild-type Col17	c.4320insC	c.4358-1G>A	Splice-site mutation restores the frameshift, beginning at amino acid 1453, and results in Col17 protein of correct size with a stretch of 13 different amino acids.
117-01	43[2]		Both hands	c.3131delC			c.3131delC		
134-01	8		Both hands, arms, back, knees, upper legs, right lower leg, and abdomen	c.1260delC			c.3495-3496delCT		
208-01	49	Two patches on right wrist	No other areas found	c.2237delG	c.2227+153_2336-318del c.2238C>T	Exon skipping of exons 30 and 31, resulting in deletion of 108-bp, which corresponds with 36 amino acids Exon skipping of 36-bp exon 30	c.2237delG		

Abbreviation: JEB-nH, non-Herlitz junctional epidermolysis bullosa.
[1]Patient numbers for which revertant mosaicism is confirmed by protein and DNA analysis are bold and underlined.
[2]Patient is deceased.

mutations, revertant areas are pigmented (for comparison see Figure 3f), whereas such difference in (hyper)pigmentation is not noticeable in patients with dystrophic EB and revertant mosaicism (Almaani *et al.*, 2010; Pasmooij *et al.*, 2010; Van den Akker *et al.*, 2011). In contrast to the generalized JEB-nH patients, in the *COL17A1* patients with a localized JEB-nH phenotype in the Netherlands, i.e., four patients in total, revertant skin areas with hyperpigmentation were not obvious. Although it cannot be excluded that *in vivo* reversion occurs in these localized JEB-nH patients, possible revertant patches will not be noticed as they will not clearly stand out, because the skin from localized JEB-nH patients is not hypopigmented as in generalized JEB-nH because of the presence of altered Col17. The phenotype, genotype, and immunofluorescence antigen mapping of three of these patients with localized disease were extensively described earlier: EB086-01, EB098-01, and EB168-01 (Pasmooij *et al.*, 2004a, b, 2007b).

To observe the revertant skin patches, a reversion event should have taken place in an epidermal stem cell, which subsequently had a positive selection advantage *in vivo*, followed by expansion leading to restoration of skin function. *COL17A1* mutations that arise, which do not result in a stable Col17 protein, will not provide any selective advantage, and therefore will remain unnoticed. The revertant area in patient EB035-01 did not noticeably change in size over a period of 3 years, suggesting that the selection advantage would be present during embryogenesis or early in childhood. For example, patient EB208-01 indicated that he had noticed the first revertant skin patch around 10 years of age. Up till now, the patients described with Col17 deficiency and revertant skin patches are all adults (Jonkman *et al.*, 1997; Pasmooij *et al.*, 2005) from whom no pictures are available just after birth. To learn more about the possible expansion of revertant skin areas at a younger age, it would be interesting to diagnose revertant mosaicism already in infancy in patients with Col17 deficiency. The two children of 8 (EB134-01) and 11 (EB084-01) years of age will be closely followed up by us (Table 1). Keratin 14, laminin-332, type VII collagen, and type XVII collagen are all expressed in epidermal stem cells, and consequently a selective advantage in revertant keratinocytes could be expected when these proteins are expressed again, or to a higher extent compared with their mutant counterparts. In an inducible mouse model for EB simplex, such a selection advantage has been shown for epidermal stem cells without the K14 mutation (Cao *et al.*, 2001). It is therefore anticipated that revertant mosaicism will also be observed in patients with mutations in other EB genes that code for proteins that are expressed by the basal keratinocytes, such as *KRT5*. No correcting somatic mutations were found in the *COL17A1* gene in fibroblast samples, which seems only logical, as Col17 is not produced by fibroblasts; thus, correction will not lead to a growth advantage, and therefore such *COL17A1* mutations will remain unnoticed.

As UV light can induce somatic mutations (Pfeifer *et al.*, 2005), this raises the question whether UV radiation has a role in inducing the correcting mutations. Although revertant patches were present in areas that are more frequently sun exposed, such as the hands, many revertant patches were also seen in areas that receive almost no sun exposure, arguing against a major role of UV exposure. Although a role cannot be excluded, indel mutations and large deletions are also not typical UV-induced mutations. Nonetheless, one might profit from the sun exposure in distinguishing the revertant skin areas, as sun exposure increases the contrast in pigmentation between revertant and mutant areas (Figure 3a and c). The hyperpigmentation of Col17 revertant patches might be explained by its pivotal role in pigment cell maintenance. *COL17A1*-null mice display a pigment defect, as the hair is white instead of black over the entire body (Nishie *et al.*, 2007). The same group recently showed that the expression of Col17 in hair follicle stem cells is required for the maintenance of melanocyte stem cells, which do not express Col17 (Tanimura *et al.*, 2011). In the revertant patches, a Col17-dependent niche is provided for the melanocyte stem cells, whereas in the nonrevertant skin areas the melanocyte stem cells will be depleted, which corresponds with the absence of Col17 formation. The fact that the laminin-332 revertant areas show (hyper)pigmentation suggests that there is also a link between laminin-332 and the melanocyte stem cells.

In the future, revertant keratinocytes might be used for obtaining a skin graft of ''naturally corrected'' cells to treat affected skin. In a study by Gostynski *et al.* (2009), adhesive stripping was proved to be effective to remove the epidermis. Functional repair was not yet achieved because of the low percentage of revertant cells in the graft. Consequently, further research into optimal growth and selection conditions for revertant keratinocytes is required before revertant cell therapy can be brought into clinical practice. Another exciting possibility is combining natural gene therapy with induced pluripotent stem (iPS) cell technology. Recently, two groups have successfully generated iPS cells from patients with recessive dystrophic EB, and subsequently differentiated these iPS cells, either spontaneously (Tolar *et al.*, 2011b) or induced (Itoh *et al.*, 2011) into keratinocytes. Patient-specific keratinocytes from revertant mosaic patches that have been corrected spontaneously could be used as a source for patient-specific iPS cells and provide an essentially unlimited number of patient-specific cells for grafting. Alternatively, iPS cells may be differentiated into both hematopoietic and mesenchymal stem cells, which can home into blistered areas of the skin following bone marrow transplantation (Wagner *et al.*, 2010; Tolar *et al.*, 2011a): ''from skin to blood cell to repair skin''. The fact that the majority, if not all, generalized JEB-nH patients with *COL17A1* mutations in the Netherlands have revertant keratinocytes makes it even more exciting to explore further therapeutic options with these naturally corrected cells.

MATERIALS AND METHODS

Biopsy sites

After written, informed consent and local ethics committee approval, and in accordance with the Declaration of Helsinki Principles, punch-biopsy specimens were obtained.

Immunofluorescence. From patient EB035-01, four biopsies were taken, from which three were from clinically unaffected skin of the

right upper leg (R1, Figure 1e), left knee (R2, Figure 1f), and right knee (R3, Figure 1g), and one from nonlesional affected skin of his right upper leg (M1, Figure 1b). From patient EB035-02, one biopsy was taken from clinically unaffected skin of the left hand (R4, Figure 2c) and one from nonlesional affected skin of the upper arm (M2). The biopsy specimens of patient EB208-01 were from clinically unaffected skin of the wrist (R5 and R6, Figure 2d and e) and from nonlesional affected skin of the right upper leg (M3). The two biopsies from patient EB025-01 were from nonlesional affected skin of the right lower arm (M4, Figure 3b) and nonlesional unaffected skin of the right lower arm (R7, Figure 3b).

Electron microscopy. Four 2-mm punch-biopsy specimens were obtained from patient EB035-01 from the same sites as the biopsies that were taken for IF microscopy (R1, R2, R3, and M1). From patient EB035-02, one biopsy was taken from nonlesional affected skin of the back and clinically unaffected skin of the left hand (R4), and from patient EB208-01 one biopsy was taken of nonlesional affected skin of the upper leg (M3) and clinically unaffected skin of the wrist (R6).

Immunofluorescence antigen mapping

mAbs for detection of Col17 were 1A8C, 1D1, NCC-Lu-226, and 233. 1A8C, 1D1, and 233 were gifts from Dr K. Owaribe, Nagoya, Japan (Di Zenzo *et al.*, 2004), whereas NCC-Lu-226 was a gift from S. Hirohashi, Tokyo, Japan (Yamada *et al.*, 1996). The fluorescence signal was quantified using the image-processing program Image J version 1.44 (http://rsbweb.nih.gov/ij/index.html). We assessed by optical comparison which part of the BMZ of a skin section was stained positively for type XVII collagen in the revertant skin biopsies.

DNA and RNA isolation

DNA recovery of revertant and mutant keratinocytes was performed as described previously (Pasmooij *et al.*, 2005). For RNA isolation and cDNA synthesis, the same methods were used as in Pasmooij *et al.* (2010).

Identification of mutations in skin samples

For detection of mutations in laser dissection microscopy–isolated DNA, we used nested PCR. All PCRs were repeated with templates from at least three separate nucleic acid isolations obtained by laser dissection microscopy. The cDNA samples from skin sections were also subjected to nested PCR. Primer sequences are listed in Supplementary Table S1 and 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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