

## ONLINE FIRST

# Natural Gene Therapy in Dystrophic Epidermolysis Bullosa

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**Background:** Dystrophic epidermolysis bullosa is a genetic blistering disorder caused by mutations in the type VII collagen gene, *COL7A1*. In revertant mosaicism, germline mutations are corrected by somatic events resulting in a mosaic disease distribution. This “natural gene therapy” phenomenon long has been recognized in other forms of epidermolysis bullosa but only recently in dystrophic epidermolysis bullosa.

**Observations:** We describe a 21-year-old man with recessive dystrophic epidermolysis bullosa carrying the homozygous c.6508C>T (p.Gln2170X) nonsense mutation who reported an unaffected skin patch on his neck where blisters never had occurred. Immunofluorescent type VII collagen staining was normal in 80% of the unaffected skin biopsy; however, it was strongly reduced in the affected skin. In the unaffected skin, the somatic nucleotide substitution c.6510G>T reverted the germ-

line nonsense codon to tyrosine (p.Gln2170Tyr), thereby restoring functional protein production.

**Conclusions:** Revertant mosaicism is considered rare in recessive dystrophic epidermolysis bullosa. However, it might be more common than previously anticipated because our patient is the third in whom revertant mosaicism was identified in a short period of time. The correction mechanism is different than that previously reported. Systematic examination of patients with recessive dystrophic epidermolysis bullosa, therefore, will likely reveal more patients with revertant patches. This is important because the natural gene therapy phenomenon may provide opportunities for revertant cell therapy.

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**R**EVERTANT MOSAICISM IS THE phenomenon in which the pathogenic effect of a germline mutation in a specific gene is corrected by a second, somatic event, thus giving rise to a mosaic appearance of mutant and revertant tissue.<sup>1</sup> This “natural gene therapy” phenomenon has been described for several genetic disorders, among which is the heritable blistering disorder epidermolysis bullosa (EB), especially the non-Herlitz junctional subtypes.<sup>2-5</sup> In fact, more than one-third of patients with non-Herlitz junctional EB who have *LAMB3* or *COL17A1* mutations displayed revertant skin patches.<sup>6</sup> As recently summarized by Lai-Cheong et al,<sup>5</sup> several different correction mechanisms have been uncovered, such as back mutations, additional nucleotide changes, insertions or deletions, and gene conversions, sometimes even within the same patient.<sup>6-8</sup> Revertant mosaicism also has been described for patients with EB simplex who have *KRT14* mutations and for a patient with Kindler syndrome.<sup>9-11</sup> However, revertant mosaicism in dystrophic epidermolysis bullosa

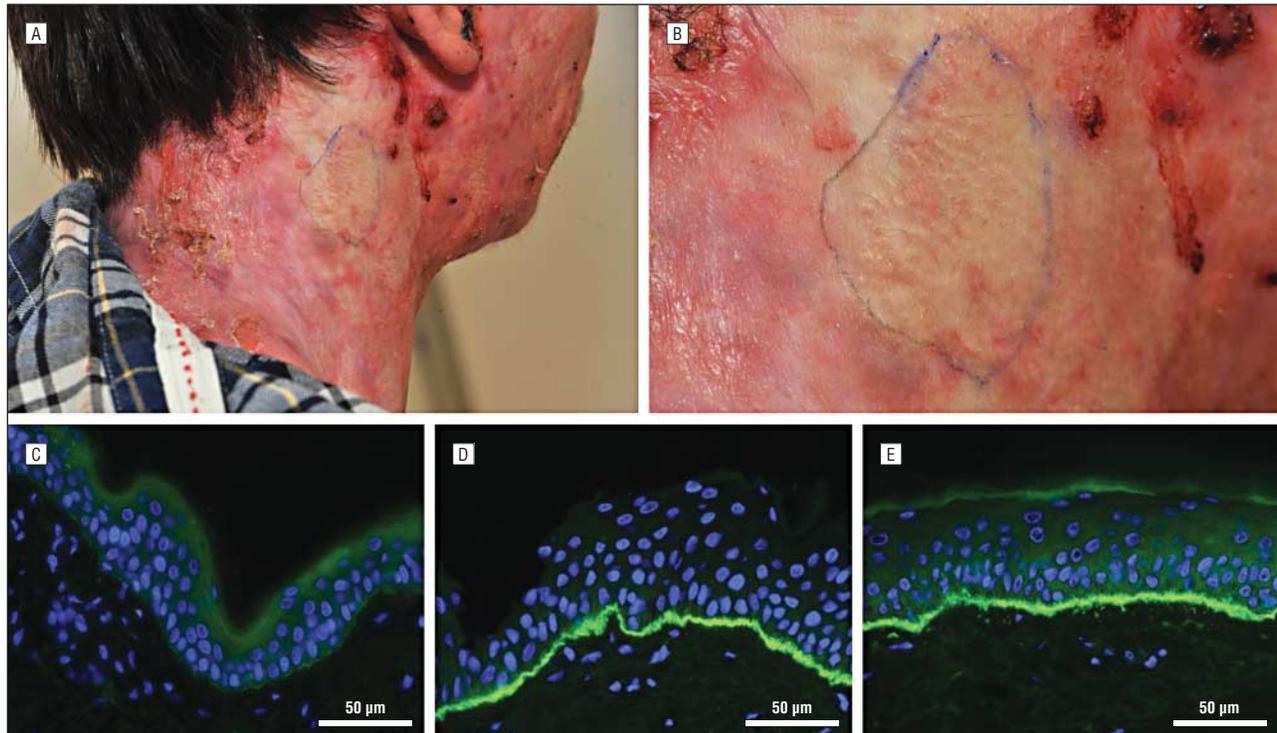
(DEB) had not been recognized until recently. Almaani et al<sup>12</sup> identified the first patient known to have recessive DEB (RDEB) with revertant mosaicism due to an intragenic crossover within the type VII collagen (*COLVII*) gene, *COL7A1*. Pasmooij et al<sup>13</sup> subsequently reported on revertant mosaicism in a patient with RDEB due to a somatic nucleotide deletion within *COL7A1*. Herein, we describe a patient with RDEB who has revertant mosaicism due to a third type of correction, a nucleotide substitution that corrects the germline nonsense codon by changing it to a tyrosine.

## REPORT OF A CASE

The patient (identification No. EB024) was first seen in our clinic at the age of 6 years, when he was diagnosed as having the generalized other subtype of RDEB,<sup>14</sup> after which the family discontinued follow-up in our clinic. At the age of 21 years, he recontacted our clinic with generalized blistering, scarring, and atrophic skin. He had developed moderate flexion contractures of the fingers and partial fusion of the fingers and toes;

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**Figure 1.** Revertant skin patch and restoration of type VII collagen staining. A, The pale revertant skin patch stands out against erythematous skin. B, Follicular skin texture. Immunofluorescent type VII collagen staining is strongly reduced along the dermoepidermal junction in affected skin (C) but normal in the revertant skin (D) compared with healthy control skin (E).

therefore, he was diagnosed as having generalized other RDEB with late-onset pseudosyndactyly (he is also patient 17 in the study by van den Akker et al<sup>15</sup>). After being asked explicitly, he reported a skin patch in his neck where, in contrast to the rest of his body, blisters had never developed spontaneously, not even after scratching. The patch had been present as long as he could remember and it had not grown. On examination, a pale skin patch of approximately 2.5 × 3.0 cm on the right lateral portion of his neck stood out from the surrounding erythematous skin that had EB (**Figure 1A**). This patch resembled unaffected skin. Small hairs and hair follicles were visible in this patch (Figure 1B).

After ethical approval by the Institutional Review Board of the University Medical Center Groningen and informed consent (in accordance with the tenets of the Declaration of Helsinki), 4-mm punch biopsy samples were collected from a nonlesional area of affected skin from the left upper leg and from the unaffected skin patch of the neck of the patient. Immunofluorescence analysis with monoclonal antibodies LH7.2 (Sigma-Aldrich Co LLC, St Louis, Missouri) and 2Q633 (United States Biological, Swampscott, Massachusetts) showed strongly reduced staining of COLVII (Figure 1C) in the biopsy sample from nonlesional affected skin, representing mutant DEB skin. The biopsy sample from unaffected skin revealed COLVII staining with the same intensity as healthy human control skin over 80% of the dermoepidermal junction (Figure 1D and E), representing revertant keratinocytes. The other 20% of the dermoepidermal junction displayed strongly reduced COLVII staining, comparable with that of mutant skin. Anchoring fibrils, as visualized by electron microscopy, were absent in mutant skin (data not shown).<sup>15</sup> A biopsy sample from the rever-

tant skin patch to be examined by electron microscopy could not be obtained.

Mutation analysis of *COL7A1* in genomic DNA from peripheral leukocytes revealed a homozygous C>T transition at nucleotide position 6508 in exon 80 (c.6508C>T) (GenBank NM\_000094.3, OMIM 120120).<sup>15</sup> This transition introduces a premature termination codon (PTC) at codon 2170 (p.Gln2170X). The breakdown of messenger RNA molecules harboring this PTC following the nonsense-mediated messenger RNA decay pathway explains the strongly reduced COLVII staining in mutant skin.<sup>16</sup> We performed laser dissection microscopic examination using the Leica LMD6000 laser microdissection microscope (Leica Microsystems GmbH, Wetzlar, Germany) on 4-µm skin cryosections from mutant and revertant skin to separate keratinocytes with normal COLVII staining from those with reduced staining and from fibroblasts.<sup>13</sup> Also, RNA was isolated from 5-µm sections of mutant and revertant skin (Qiagen RNeasy Plus Micro Kit; Qiagen NV, Venlo, the Netherlands), and complementary DNA (cDNA) was synthesized as described.<sup>13</sup> Subsequently, nested polymerase chain reaction assays were performed on genomic DNA and cDNA (for primers, see the study by Pasmooij et al<sup>13</sup>). As expected, mutant keratinocytes carried the homozygous c.6508C>T transition (**Figure 2A**). However, in addition to this germline mutation, mutation analysis of revertant keratinocytes disclosed the extra heterozygous transversion c.6510G>T that was not present in keratinocytes from mutant skin or in fibroblasts from either type of skin. This somatic mutation is located 2 nucleotides downstream from the germline c.6508C>T mutation and corrects the PTC at codon 2170 on 1 allele by changing it to tyrosine (Figure 2B). Hence,

in his revertant skin patch, our patient is functionally homozygous for the missense change p.Gln2170Tyr. The c.6510G>T reversion mutation also was present in cDNA of revertant keratinocytes (not shown).

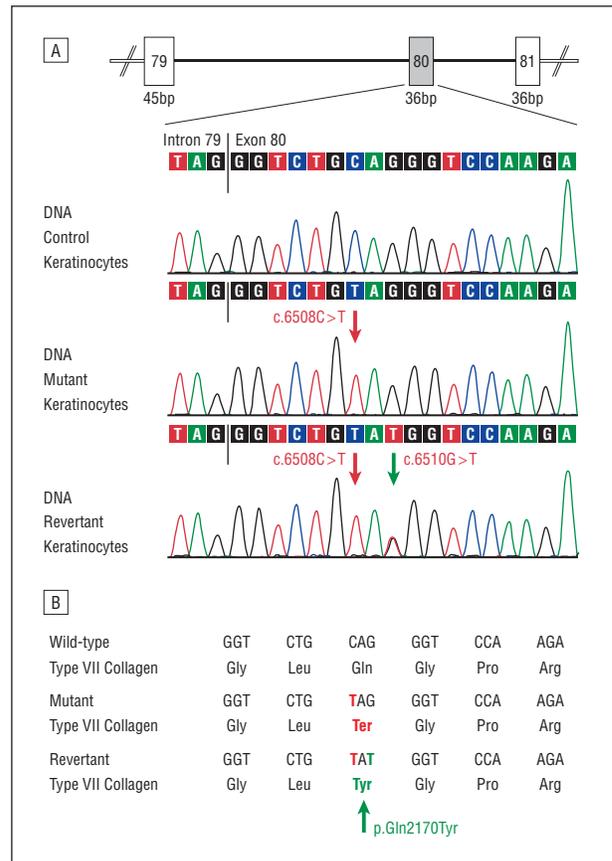
## COMMENT

For many years, revertant mosaicism, also known as natural gene therapy, resulting from spontaneous somatic correction events was observed almost exclusively in the non-Herlitz junctional subtypes of EB.<sup>1,2,6-8</sup> Although occasional case reports have appeared describing revertant mosaicism in patients with autosomal dominant EB simplex,<sup>9</sup> autosomal recessive EB simplex,<sup>10</sup> and Kindler syndrome,<sup>11</sup> revertant mosaicism was not identified in DEB, which raised the question of whether it did not occur in DEB or whether it simply was not recognized in patients with DEB. With the recent identification of revertant mosaicism resulting from spontaneous somatic correcting events in *COL7A1* in 2 patients with RDEB,<sup>12,13</sup> it was shown that natural gene therapy occurs in all 4 major EB types, including DEB.

The identification of revertant mosaicism in the patient with RDEB described herein suggests that somatic correcting events are more common in *COL7A1* than previously anticipated. It is probable that revertant skin has been overlooked in other patients with RDEB in the past because it was not recognized as such. To date, in the reports of patients with RDEB who have revertant mosaicism, the revertant skin patches stood out as pale areas surrounded by erythematous eroded skin.<sup>12,13</sup> Systematic examination of patients with RDEB for similar patches, therefore, will likely disclose revertant skin in more patients.

In future studies, the identification of additional patients with RDEB who have revertant mosaicism will reveal the absolute frequency of natural gene therapy occurring on *COL7A1* alleles. This will answer the question of whether somatic correction events of mutant alleles are as common in *COL7A1* as in *LAMB3* and *COL17A1*, which are found in more than one-third of Dutch patients with non-Herlitz junctional EB.<sup>6</sup> Also, it will reveal more information regarding the intriguing and as-yet-unsolved question of whether the *LAMB3* and *COL17A1* genes in keratinocytes are more prone to somatic mutations than are the other EB-associated genes.

The reasons that correcting mutations are frequently observed in EB genes, including *COL7A1*, remain elusive. Also, it is unclear at what exact time point they occur. A role for UV exposure or other environmental factors can be hypothesized because UV has the potential to induce somatic DNA mutations.<sup>17</sup> If such exogenous factors are the cause, the mechanism of revertant skin patch development would consist of postnatal correction mutation induction in 1 stem cell giving rise to an expanding patch of healthy skin because of positive selection. Although this mechanism might apply to some patients, it seems unlikely in our patient because his revertant skin had maintained unchanged shape and size for as long as he could remember. Also, because it has been shown that epidermal stem cells populate only small skin areas by the formation of epidermal proliferative units,<sup>18</sup> it seems more likely that the correction mutation arose in earlier stages of embryonic epidermal stem cell development. Whether the occurrence of correcting mutations is random



**Figure 2.** Correction of the germline mutation in revertant keratinocytes. A, Scale representation of the genomic area around *COL7A1* exon 80. The c.6508C>T transition is present homozygously in mutant keratinocytes. An additional heterozygous c.6510G>T transversion resides in the germline-mutated codon in revertant keratinocytes. B, This somatic mutation corrects the germline nonsense codon by changing it to tyrosine. The net effect is the missense mutation p.Gln2170Tyr. bp Indicates base pair.

or is driven by genetic or nongenetic factors, as suggested for *KRT10* frame-shift mutations causing ichthyosis with confetti,<sup>19</sup> remains a topic for further research.

The patient described herein displays a novel correction mechanism in the *COL7A1* gene. Hence, different correcting mechanisms occur in *COL7A1*, as in *COL17A1* and *LAMB3*,<sup>5</sup> whereas in patients with ichthyosis with confetti due to dominant *KRT10* mutations, the same correction mechanism of mitotic recombination occurs in each revertant skin spot in each patient.<sup>19</sup> In our patient, a somatic nucleotide change in the germline mutated codon rescues the mutant phenotype by reverting the nonsense codon to a tyrosine (p.X2170Tyr), which leads to the restoration of functional protein production. The resultant missense change p.Gln2170Tyr has been described neither as a neutral polymorphism (dbSNP, <http://www.ncbi.nlm.nih.gov/projects/SNP/>) nor as a pathogenic mutation in patients with RDEB (International Dystrophic Epidermolysis Bullosa Patient Registry, [www.deb-central.org](http://www.deb-central.org), accessed on June 30, 2011).<sup>20</sup> This missense change resides at the third position of a Gly-X-Y triplet and, contrary to substitutions of glycine residues at the first position of the Gly-X-Y triples,<sup>21</sup> it is difficult, if not impossible, to predict the effect of such amino acid substitutions on triple-helix stability and clinical outcome. The pathogenicity prediction software pack-

age Alamut, version 2.0 (Interactive Biosoftware, Rouen, France), classifies the p.Gln2170Tyr missense change as being of unknown pathogenicity. As indicated by the normal immunofluorescence staining and negative history of blistering in the revertant patch, the missense change does not seem to severely affect COLVII functioning. Altogether, it remains unknown whether the resultant p.Gln2170Tyr change affects triple-helix stability, but a mild pathogenic effect cannot be excluded.

Identification of revertant skin patches in patients with RDEB is important because revertant skin can be regarded as that in which gene therapy has been performed successfully by nature itself. This finding renders promising therapeutic opportunities, such as revertant cell therapy, namely, grafting of ex vivo-grown reverted keratinocytes onto affected skin.<sup>22</sup> Keratinocytes and fibroblasts can be reprogrammed into induced pluripotent stem cells (iPSCs) and subsequently differentiated into keratinocytes, providing an unlimited source of autologous keratinocytes.<sup>23</sup> Recently, 2 groups described the generation of iPSCs from the cells of patients with RDEB and the subsequent spontaneous<sup>24</sup> and directed<sup>25</sup> differentiation of these iPSCs into keratinocytes. The use of revertant cells obviates the need for potentially dangerous virus-mediated gene correction<sup>26</sup> and circumvents the risk of immunologic rejection, and, if combined with the patient-specific iPSCs approach, provides the opportunity to grow a large number of healthy skin grafts. Moreover, differentiation of revertant iPSCs into hematopoietic or mesenchymal stem cells would provide an autologous alternative for allogeneic bone-marrow stem cell transplantation in this genetic disease.<sup>27,28</sup> Altogether, revertant skin patches offer exciting future therapeutic opportunities to patients with EB who have received this natural gene therapy.

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**Author Contributions:** Drs van den Akker, Hofstra, Jonkman, and Pasmooij had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. **Study concept and design:** van den Akker, Jonkman, and Pasmooij. **Acquisition of data:** van den Akker, Nijenhuis, Meijer, and Pasmooij. **Analysis and interpretation of data:** van den Akker, Hofstra, Jonkman, and Pasmooij. **Drafting of the manuscript:** van den Akker and Pasmooij. **Critical revision of the manuscript for important intellectual content:** van den Akker, Nijenhuis, Meijer, Hofstra, Jonkman, and Pasmooij. **Administrative, technical, and material support:** Nijenhuis, Meijer, and Pasmooij. **Study supervision:** van den Akker, Hofstra, Jonkman, and Pasmooij.

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