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## Revertant cell therapy for epidermolysis bullosa

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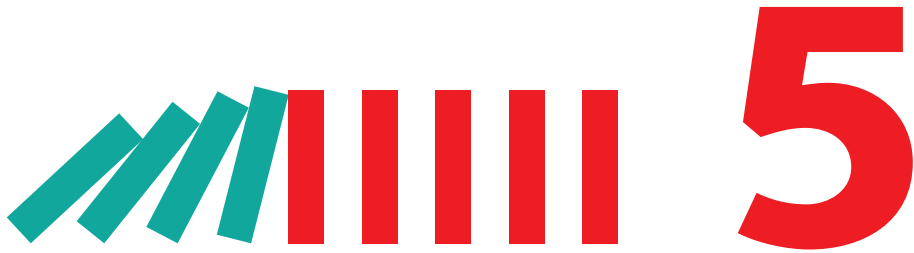
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## **PIGMENTATION AND MELANOCYTE SUPPLY TO THE EPIDERMIS DEPEND ON TYPE XVII COLLAGEN**

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## ABSTRACT

Genetic deficiency of type XVII collagen (Col17), laminin-332, or type VII collagen causes epidermolysis bullosa (EB). Spontaneous correction of the deficiency, also known as revertant mosaicism, is caused by a second somatic mutation that restores protein expression resulting in clinically healthy (revertant) patches surrounded by fragile (mutant) skin. Interestingly, in some patients patches of revertant skin show hyperpigmentation. To study the possible role of affected proteins in pigmentation and melanocyte distribution, we investigated clinical documentation and skin biopsy specimens of 13 revertant EB patients having correcting mutations in the *COL17A1*, *LAMB3*, or *COL7A1* genes.

Analysis revealed that lack of Col17 led to decreased melanin intensity and melanocyte density in the epidermis when compared to the revertant patches. Reversions of *LAMB3* and *COL7A1* in keratinocytes did not influence clinical pigmentation or density of melanocytes. We conclude that in human skin, melanocyte supply to the epidermis depends on Col17 expression in keratinocytes.

## BACKGROUND

Melanocytes are known as melanin producing cells in the epidermis and hold other functions, for example neuroendocrine in the brain and metabolic on retinoids in the eye.<sup>1</sup> In the skin, melanocytes are derived from the neural crest during embryonic development. In the interfollicular epidermis (IFE) melanocytes are not attached by hemidesmosomes, but by poorly defined dense plaques.<sup>2</sup> Resting melanocytes do not synthesize adhesion proteins such as type XVII collagen (Col17), laminin-332 (lam-332) or type VII collagen (Col7).<sup>2,3</sup>

Col17, lam-332 and Col7, along with 11 other macroproteins, are synthesized by keratinocytes and involved in the group of genetic blistering diseases called epidermolysis bullosa (EB).<sup>7-9</sup> Revertant mosaicism (RM) refers to the coexistence of cells carrying the original germline mutation and cells that spontaneously have corrected the germline mutation by a somatic reverse mutation in one individual<sup>4</sup>. This reverse mutation leads to a clinically healthy phenotype. In the last 15 years RM has been identified for five EB genes – *COL17A1*, *LAMB3*, *COL7A1*, *FERMT1*, and *KRT14*.<sup>11-14</sup> Reversion in EB has only been observed in keratinocytes and not in fibroblasts, melanocytes or peripheral blood cells.<sup>5,6</sup> The first patient with RM in EB (patient 026-01) described by Jonkman et al. had a generalized subtype of non-Herlitz junctional EB (JEB-nH-gen) due to *COL17A1* mutations.<sup>7</sup> She had a revertant (healthy) patch on her hand due to a gene conversion. This patch was more pigmented than the surrounding, mutant (affected) skin. Interestingly, revertant skin manifested as hyperpigmented patches in several patients.<sup>8,9</sup>

## QUESTIONS ADDRESSED

The spontaneous coexistence of two different microenvironments, that is mutant and revertant epidermis, within one individual may help to answer, if pigmentation and melanocyte biology in human skin depend on a protein of the epidermal basement membrane zone. We have performed a correlational study in a population of EB patients with RM for *COL17A1*, *LAMB3* and *COL7A1* and based on our results and current literature formulated a hypothesis about importance of Col17 for melanocyte supply.

## EXPERIMENTAL DESIGN

We identified 13 patients with RM in the Dutch and Spanish EB databases: ten with JEB-nH-gen due to mutations in *COL17A1* (n=8) or *LAMB3* (n=2), and three with recessive dystrophic epidermolysis bullosa (RDEB) due to mutations in *COL7A1* (Table 1). As a control group we selected six biopsies of healthy Caucasian individuals (Table 1). Furthermore, four localized JEB-nH patients (JEB-nH-loc) were included, because of their intermediate level of Col17 expression.<sup>10</sup>

Clinical phenotype and pigmentation of mutant and revertant skin were assessed. Additionally, biopsies of all patients and control individuals were stained for Col17, lam-332, Col7, melanocytes and melanin (Supplementary).

## RESULTS

In the *COL17A1* revertant patient group all patients showed hyperpigmentation of revertant skin (Table 1). The patches were sharply demarcated by pigmentation with characteristics of healthy skin with normal skin texture (Figure 1A). Dermatoscopy aided clinical differentiation between revertant skin and EB naevi (Figure S1A-C). In contrast, revertant patches in both *LAMB3* and *COL7A1* patients showed no clear difference in pigmentation. Their revertant skin could be distinguished from the surrounding affected skin by lack of erythema (Figure 1B and 1C). Fontana-Masson staining for pigment corrals (Figure 1E and S1E) in *COL17A1* revertant patients showed more pigment present in the revertant patches than in the mutant skin (Figure S1G). Such a trend was not observed in the two other groups (Supplementary). Immunofluorescence staining revealed a lower density of melanocytes in the mutant skin (median: 2.60 melanocytes/1000 $\mu$ m basement membrane; Figure 1D, 1F, 1G, S1D and S1F), when compared with revertant skin (median: 9.41) of *COL17A1* revertant patients. Surprisingly, *LAMB3* revertant patients showed a lower density of melanocytes than the control group, yet no difference was found between revertant (median: 4.38) and mutant (median: 4.23) skin (Supplementary). Taken together, pigmentation and melanocyte density were significantly lowered only in Col17 deficient JEB-nH-gen skin.

## DISCUSSION

Col17 is a transmembrane hemidesmosomal protein synthesized in the skin by keratinocytes.<sup>11,12</sup> This study demonstrates that lack of Col17 significantly impairs melanisation and decreases the number of melanocytes in skin ( $p < 0.01$ ), whereas restoration of Col17 expression in revertant skin results in normal melanisation and melanocyte density. Our work confirms that revertant patches in Col17 deficient patients can clinically be recognized by hyperpigmentation. Interestingly, we found normal melanocyte density in the skin of JEB-nH-loc patients meaning that slightly reduced Col17 levels are sufficient for normal melanocyte distribution. Therefore, the amount of Col17 needed for maintaining the melanocyte population seems to be lower than that needed for maintaining a healthy dermal-epidermal junction.

Recent findings of the important role of Col17 in melanocyte stem cell survival, cell signalling and immune response might explain loss of melanocytes in Col17 negative skin. Nishie et al.<sup>13</sup>

and Tanimura et al.<sup>3</sup> showed hair greying and alopecia in Col17 <sup>-/-</sup> mice due to hair follicleatrophy. Tanimura et al. explained how hair follicle stem cells (HFSCs) and melanocyte stem cells (MeSCs) interact with each other in murine hair follicles.<sup>3</sup> Lack of Col17 expression results in reduced paracrine stimulation of MeSCs with TGF- $\beta$  and leads to follicle atrophy due to depletion of both HFSCs and MeSCs.<sup>6,23,24</sup> As universal alopecia is also characteristic in completely Col17-deficient JEB-nH-gen patients, the same mechanism might be responsible for melanocyte depletion in Col17 mutant skin. The recent work of Van den Bergh et al.<sup>11</sup> showed that Col17 is involved in the immune response. Col17 negative keratinocytes had much higher levels of NF- $\kappa$ B than Col17 positive cells. This leads to overstimulation and an increased IL-8 response, which can attract T-cells and promote autoimmune targeting of melanocytes. IL-8 together with IL-6 can also directly inhibit melanocyte growth and modulate their antigen expression.<sup>14-16</sup>

All studied patients with *LAMB3* mutations had a lower than normal density of melanocytes and no melanin in both revertant and mutant skin. Reversion of keratinocytes in healthy patches did not rescue the melanocytes, as we saw in the *COL17A1* revertant group. Lam-332, in contrast to Col17, can be synthesized and secreted by actively dividing melanocytes and it has been earlier implied that lam-332 might be important for melanocyte proliferation.<sup>2</sup> This could possibly explain the lower density of melanocytes in patients with *LAMB3* mutations, as melanocytes are not revertant and thus not able to express lam-332 in *LAMB3* revertant and mutant skin. Further, our results suggest that Col7 is not directly involved in melanocyte biology as melanocyte distribution and pigmentation in both revertant and mutant *COL7A1* skin were normal.

In conclusion, we have proved that hyperpigmentation is a sign of RM in patients with mutations in *COL17A1*. Moreover, we show that there is a correlation between the levels of Col17 expression and presence of melanocytes in human epidermis, which deem further studies necessary to understand the underlying mechanism.

Phenotypic differences												
Affected gene	Clinical diagnosis	Patient	Difference in pigmentation between mutant and revertant skin	Hair growth		IF of the mutant skin				Reference		
				Scalp hair	Body hair	Type XVII Collagen (1A8C)	IC (Lu-226/233)	EC	Laminin-332		Type VII collagen	
COL17A1	JEB-nH-gen	025-01	Yes	Absent	Absent <sup>1</sup>	-	-/+	+	+	+	9,17	
		026-01	Yes	Absent	Absent	-	-/+	+	+	+	8,10	
		035-01	Yes	Absent	Absent	-	-/+	+	+	+		
		035-02	Yes	Sparse	Absent <sup>1</sup>	-	+	+	+	+		
		093-01	Yes	Sparse	Absent	-	+	+	+	+		
		134-01	Yes	Normal	Absent <sup>1</sup>	-	+	+	+	+		
		208-01	Yes	Normal	Yes	Normal	-	+	+	+	8	
		248-01	Yes	Absent	Yes	Absent	-	ND	+	+	+	in preparation
		086-01	N/A	Normal	N/A	Normal	-/+	+	+	+	+	10
		098-01	N/A	Normal	N/A	Normal	Reduced	-/+	+	+	+	
LAMB3	JEB-nH-gen	168-01	N/A	Normal	Normal	-/+	+	+	+	+		
		224-01	N/A	Normal	Normal	ND	+	+	+	+		
		029-01	No	Normal	Reduced	+	+	-/+	+	+	5	
		078-01	No	Normal	Reduced	+	+	-/+	+	+		
COL7A1	RDEB	024-01	No	Normal	Normal	+	+	+	+	-/+	18	
		260-01	No	Normal	Absent	+	+	+	+	-/+	19	
		265-01	No	Normal	Absent	+	+	+	+	-	in preparation	

**Table 1:** Clinical phenotype of included patients related to if antigen mapping of their mutant skin

**Table legend:** N/A – not applicable; ND – not determined; - negative; -/+ reduced; + positive; IC – intracellular domain of Col17; EC – extracellular domain of Col17; 1 single body hair growth observed on revertant patches.

## SUPPLEMENTARY

### Material and methods

#### Patients

We identified 13 EB patients with RM in the Dutch and Spanish EB registry database: ten with JEB-nH- gen *COL17A1* (n=8) and *LAMB3* (n=2), and three with recessive dystrophic epidermolysis bullosa (RDEB) due to mutations in *COL7A1*. As a control group we selected six biopsies of healthy Caucasian individuals, three taken from sun exposed skin and three from non-exposed skin. Furthermore, four localized JEB-nH patients (JEB-nH-loc) were selected, because of their reduced, but not absent, intermediate level of Col17 expression (10). Clinical phenotype and pigmentation assessments were based on information from EB- patient database, clinical photography and previous publications. The 4-6 mm diameter punch biopsies, taken previously from mutant and revertant skin of EB patients (5,7-10,19), mutant skin of JEB-nH-loc patients and healthy skin of control patients, were re-sectioned, stained and examined for this study. All biopsies used had been stored at -80C. Informed consent and approval of the local ethical committee was in accordance with the Declaration of Helsinki Principles.

#### Analysis of skin sections

We used the following monoclonal antibodies (mAbs) against Col17: 1A8C to the intracellular domain, 1D1 and 233 mAbs to the extracellular domain (all gifts from Dr. K. Owaribe, Nagoya, Japan)<sup>20</sup> for diagnostic assesment (Table 1) and VK5 to the intracellular domain (dr. H. H. Pas, Groningen, the Netherlands) for double staining with melanA; against lam-332: GB3 (Abcam) to  $\gamma$ 2 chain; against Col7: LH7.2 (gift from I. Leigh, London, United Kingdom), and against the melanocyte marker melan-A: M2-7C10. Sections of 4- $\mu$ m thickness were cut and air-fixed. VK5, LH7.2 and GB3 were conjugated with Zenon® Mouse IgG Labeling Kits AlexaFluor® 488 (green) and M2-7C10 with AlexaFluor® 568. mAbs were diluted in PBS/OVA 1% and sections were incubated at room temperature for 45 min. Hoechst 33342 was used to stain nuclei. In each section melan-A positive melanocytes were counted under 63x magnification, and expression of proteins was assessed by optical comparison with control sections. The basal cell layer in all sections was photographed along the complete length of the specimen, and pictures of sectors were merged with Adobe Photoshop CS3 software. The length of the basal membrane was determined with image- processing software - ImageJ, version 1.44 (<http://rsbweb.nih.gov/ij/index.html>). The sections used for IF antigen mapping were incubated in PBS for 30 min to remove the cover glass, dried, and stained with Fontana-Masson to reveal melanin in the same section. Stained sections of each patient were scored semi-quantitatively by an experienced pathologist (section identity was blinded): 0 – no melanin, 3 – maximal amount of melanin, 0.5 step. The binominal test with p=0.5 was used to test statistical significance.



## Results

### Dermoscopy of Col17 revertant skin

In addition to the aforementioned results, in *COL17A1* revertant patients dermoscopic examination could be used to easily distinguish revertant skin (Figure S1A-C). Mutant skin presents a homogenous image with dotted vessels and no pigment network (Figure S1A), while revertant skin was identifiable by pseudo-pigment network with follicular pigmentation (Figure S1B). Patients 134-01 and 025-01 also had EB naevi, which can present different patterns, i.e. globular or cobblestone pattern as in case of these two patients (Figure S1C).<sup>21</sup>

### Pigment staining

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Differences in intensity of melanin between revertant and mutant skin were compared for each patient separately (Figure S1G). In the *COL17A1* mosaic group significantly more pigment was seen in all of the tested revertant skin than in their corresponding mutant skin (positive values in Figure S1G). Interestingly, in revertant biopsies where co-existence of revertant and mutant basal keratinocytes in one skin section could be seen, pigment allocation did not co-distribute with restored protein expression (Figure S1D-E). In the *COL7A1* revertant group, no correlation could be discerned between revertant and mutant skin. In all sections of *LAMB3* patients melanin was absent.

### Melanocyte density

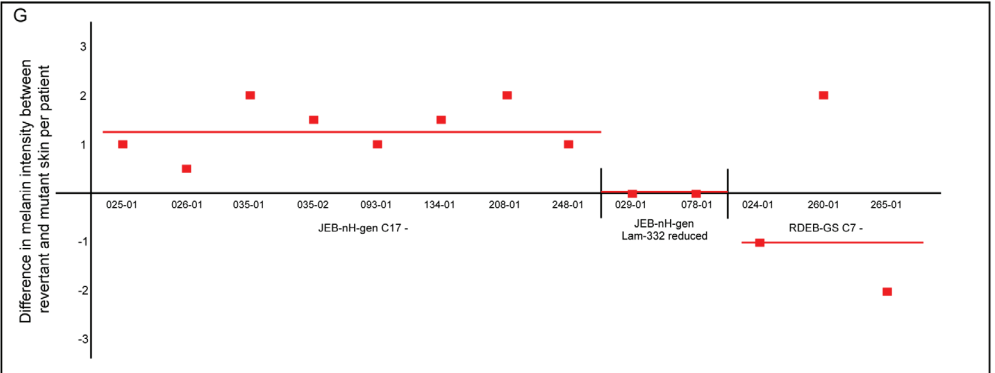
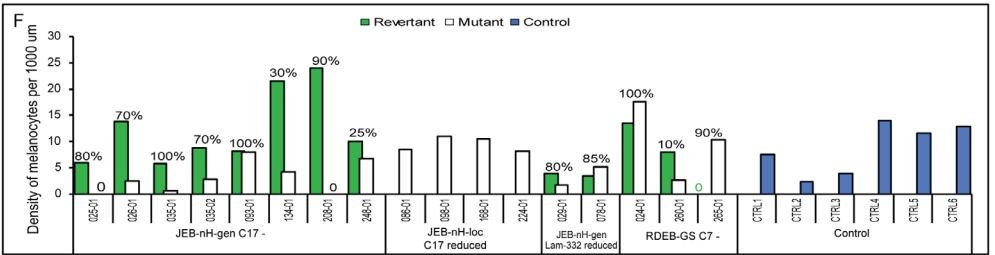
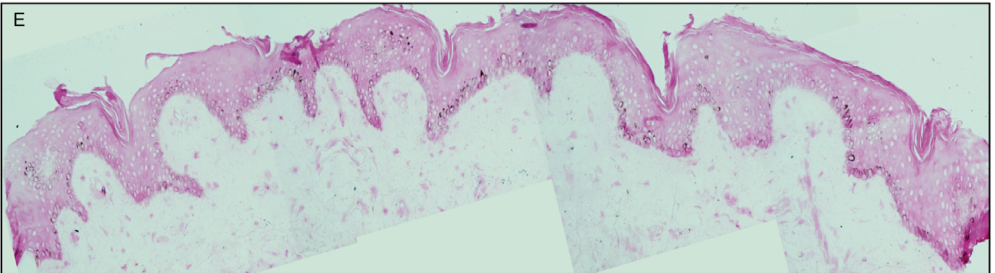
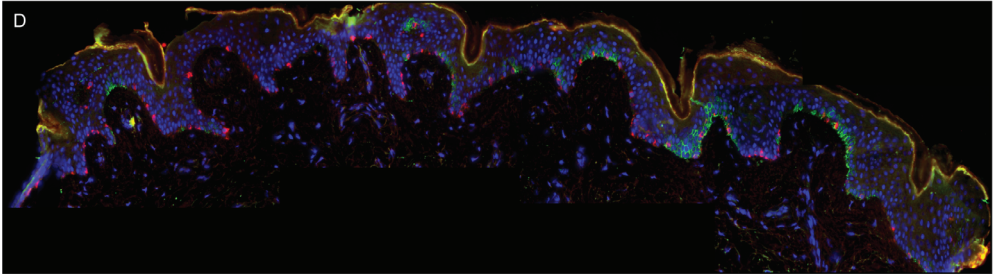
Median density of melanocytes in control group was 9.60 per 1000  $\mu\text{m}$  of basement membrane, which corresponded to the melanocyte-keratinocyte ratio of 1/10.4. Patients mosaic for *COL17A1* presented with a density of melanocytes in their revertant skin (median: 9.41, ratio: 1/10.5) almost identical to our control group. Melanocytes were distributed evenly in IFE of each section and did not co-distribute with micro-mosaicism for *COL17A1* (Figure 1g and S1d). Their mutant, Col17 deficient, skin showed however a significantly lower melanocyte density (median: 2.60, ratio: 1/38.5). The four patients with JEB-nH-loc showed very similar results as control and *COL17A1* revertant group (median: 9.56, ratio: 1/10.5). *COL7A1* revertant patients had a normal number of melanocytes in mutant skin (median: 10.38, ratio: 1/9.5), and this number was slightly lower in revertant skin (median: 8.05, ratio: 1/12.5), but still within the normal values.

### **Micro-mosaicism**

Skin sections of revertant skin often show micro-mosaicism, a coexistence of short segments of revertant and mutant keratinocytes within one slice, which we observed in 10 of 13 revertant biopsies (Figure 1D, S1D and S1F). When considering the influence of Col17 on melanocytes, we expected that in *COL17A1* revertant skin sections melanocytes would co-localize with revertant segments. However, melanocytes in these sections were evenly distributed throughout both the revertant and mutant segments. This might suggest a paracrine effect of Col17 on melanocytes in the human epidermis.

**Figure S1.** Dermoscopy of patients 134-01: a) mutant skin on the right wrist, b) revertant skin on the left arm and c) EB naevus. In mutant skin hypopigmentation and dotted vessels are seen, in the revertant skin a pseudo-pigment network and follicular pigmentation are clearly visible. EB naevus shows globular pattern with irregular dots. d) IF antigen mapping of Col17 in green, melan-A in red, and nuclei in blue. e) Identical section re-stained with Fontana-Masson (dark pigment grains). f) Melanocyte density (Y-axis: melanocytes/1000  $\mu$ m BMZ) per patient, number above green bars shows the percentage of revertant cells (micro-mosaicism) within the biopsy. Green depicts revertant patients, white depicts mutant patients and blue depicts controls. g) Difference in melanin intensity (Y-axis, -3 to 3 value) per patient. Each square represents difference between revertant and mutant skin, positive result represents more pigment in revertant patch, while negative result represents more pigment in mutant patch. Red lines show median values per patient group.

# 5



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