Revertant cell therapy for epidermolysis bullosa

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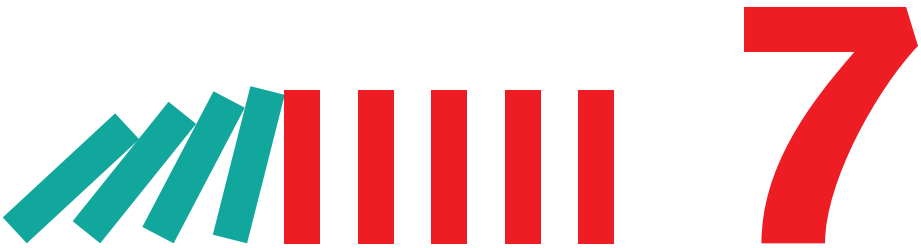

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

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REVERTANT CELL THERAPY: CURRENT STATE OF THE ART

How far are we?

The proof-of-principle of revertant cell therapy was demonstrated in **Chapters 2-4** of this thesis. Different techniques can be used to expand an area of healthy skin. In **Chapter 2** we showed that autologous transplantation of cultured epithelial sheets is feasible in a patient, although survival of revertant keratinocytes during graft production was insufficient. In **Chapter 3** we showed that production of cultured skin grafts could be improved and that long-term survival of revertant cells was possible in a mouse model. Finally, in **Chapter 4** we described the successful, long-term acceptance of revertant punch grafts was described as a cure of persistant ulcers in EB. Therefore, if a sufficient area of revertant skin is available, as in our patient with Lam-332 deficiency described in **Chapter 4**, it is currently possible to treat crucial affected areas with non-cultured autologous revertant skin grafts. This method is simple, does not require a special laboratory and is available to most patients with revertant mosaicism, depending on the size and localization of their healthy skin patch.

Advantages of the current approach

If one looks at the therapeutic approaches described in **Chapter 1** of this thesis, one will see that these are based on allogeneic material (protein therapy and cell therapy) or genetic manipulation (gene therapy). The most important value of revertant cell therapy is the use of autologous, naturally corrected cells.[1,2] It avoids the need to introduce allogeneic material, as in the cell therapy approaches, or to manipulate the genome, as required by gene therapy. Contrary to these methods, revertant cell therapy has no risk of inducing an autoimmune response or off-target effects in the genome that may induce neoplasia. As revertant cell therapy uses naturally corrected keratinocytes, it could be considered natural gene therapy combined with cell therapy.

Advantages	Disadvantages	Possible limitations
Autologous material	Limited number of revertant patients available	Depletion of revertant stem cells
No genetic manipulation	Limited expansion ratio of punch grafting	Higher mutation ratio in revertant cells
No auto-immune response		

Table 1. Advantages, disadvantages and possible limitations of the current approach to revertant cell therapy

Concerns and possible limitations of the current approach

As the advantages of revertant cell therapy seem to be indisputable; possible limitations and disadvantages should always be considered. First of all, revertant cell therapy can only be applied in patients with identified revertant mosaicism. At the moment it is expected that all of the JEB-gen intermed patients with mutations in *COL17A1* have visible revertant mosaicism and recently revertant mosaicism has been identified in a number of DEB patients.[3,4] We are, however, far from the statement that all patients with JEB and DEB have identifiable revertant mosaicism, as in many patients revertant patches are yet to be found. The age of patients can also limit the *in vitro* expansion, as it is normal for all humans to deplete the stem cell population with age.[5] Moreover, in the form proposed in **Chapters 2-4**, revertant cell therapy aims to only treat cutaneous manifestations of EB. With this being a disadvantage, as the treatment of the all affected organs should be the ultimate aim of a successful therapy for a genetic disease, one should not forget that most of the therapies currently under development are able to provide treatment of all the manifestations of EB.[6-10]

The biology of revertant mosaicism is not yet fully understood. For example it is believed that revertant keratinocytes are simply a healthy population of cells in an affected body and thus do not have a higher mutation rate than other corresponding cell populations.[11,12] Mutation rates in revertant cells have been determined in revertant mosaic patients with Wiskott-Aldrich syndrome (WAS), and was found to be normal,[12,13] which is a strong argument for the above stated assumption.

Depletion of the growth potential of revertant keratinocytes could also become a potential threat to revertant cell therapy. In the first successful gene therapy for EB, Mavilio et al. described problematic *in vitro* expansion of lam-332 deficient keratinocytes due to stem cell depletion.[14] These data suggest that because of extensive blistering, stem cells have to divide more often than in a healthy individual and thus their growth potential when found in cultured skin grafts is hereby impaired. One could speculate if the revertant cell population in EB patients becomes depleted and if this is a reason for in vitro growth disadvantage of revertant keratinocytes. In patients with WAS, X-linked SCID and adenosine deaminase deficiency exhibiting revertant mosaicism, a selective growth advantage *in vivo* of revertant lymphocytes has been observed.[15-18] If one assumes, that the same applies to revertant keratinocytes *in vivo*, the fact that revertant patches in EB patients seem to be stable in most of the patients after infancy could mean that the growth potential of revertant keratinocytes has already met their biological limit. When we take into account that *in vitro* keratinocytes can divide approximately 50 times,[19] which is in accordance with the Hayflick limit,[20] one could wonder if the difficulties in culturing revertant keratinocytes described in **Chapters 2 and 3** come from a specific growth advantage of Col17 negative keratinocytes *in vitro* and not from a depletion of growth potential of revertant cells.

The fact that long-term survival of revertant cells is achieved and that revertant cells have sufficient colony forming potential, **(Chapter 3)** together with the lack of blistering in revertant skin and success of transplantation of revertant skin **(Chapter 4)**, are strongly against depletion of revertant stem cells. Although both the higher mutation rate of revertant cells and depletion of revertant stem cells seem improbable, experiments to analyse the mutation rate and telomere length of revertant keratinocytes would certainly give clarification.

REVERTANT CELL THERAPY: EXPANDING CURRENT APPROACH

Future of revertant keratinocytes without in vitro expansion

In **Chapter 4** we presented the use of punch grafting for transplantation of revertant skin. An advantage of split thickness punch grafting lies in the small size of the biopsies that can easily be harvested from every part of the body leaving enough surrounding skin in the donor site to ensure healing with the remaining revertant skin. Patients with revertant mosaicism of the skin have single or multiple revertant patches, randomly distributed, with irregular shape and a diameter seldom larger than 5 cm, making the use of a dermatome to harvest traditional split skin grafts difficult. Furthermore, the aim to expand a revertant area means that the revertant donor site should also heal with the healthy epidermis. Harvesting a larger graft from one location might give the mutant cells an opportunity to repopulate the donor site.

The disadvantage of punch grafting is the relatively poor expansion ratio, which is typically not more than 1:4. The maximal treated area depends thus directly on the size of donor revertant patches. To achieve higher expansion from a single revertant patch, a different method of non-cultured epidermal grafting could be applied. Currently, when non-cultured epidermal grafting is considered, the highest expansion ratio can be achieved with the enzymatic digestion of small skin biopsies and application of isolated cells in suspension.[21-23] Such a method, proved successful in the treatment of burn wounds and vitiligo, can be applied during a single procedure in the operating theatre omitting the need for a GMP certified laboratory. In **Chapter 6** we described an attempt to treat a chronic ulcer in a JEB-gen-intermed patient with a commercial kit ReCell® (Avita Medical), which allows to digest a small skin biopsy and produce a skin cell suspension ready for transplantation.[24] The ReCell® manufacturer states that the kit allows an expansion rate of 1:80, meaning that from a 1cm^2 biopsy 80 cm^2 can be treated.[24] Our attempt described in **Chapter 6** was unsuccessful probably due to an impaired wound healing in the treated region. Approach with non-cultured epidermal cells in suspension should be however studied in the future in the more controlled environment. We believe that in combination with adhesive tape preparation of the wound bed, as described in **Chapter 2**, it will be an attractive solution for revertant cell therapy.

Towards successful in vitro expansion of revertant cells

In **Chapter 2** we showed the first attempt to expand revertant skin area with transplantation of cultured epidermal grafts in a patient affected by JEB-gen-intermed due to mutations in *COL17A1* and revertant mosaicism. Although the transplantation procedure and healing process were uncomplicated, we did not achieve restoration of the phenotype due to a low percentage of revertant keratinocytes in the graft. The original skin biopsy consisted of about 50% revertant cells and in the graft only 3% of the cells expressed Col17. In **Chapter 3** we again used a skin biopsy from the same patient, which also showed 50% of revertant keratinocytes at the start of the experiment. During the course of *in vitro* expansion and graft production, the percentage of Col17 revertant cells dropped to 20% and stayed at this level at least for 16 weeks after transplantation on the back of nude mice. It is therefore the *in vitro* phase that is responsible for the declining percentage of revertant keratinocytes. We will now speculate on the possible reasons for such shrinkage of the revertant population in experiments described in **Chapters 2 and 3**. With depletion of stem cells not being a convincing theory to explain the shrinkage of the revertant cell population relative to the mutant population *in vitro*, as explained earlier in this chapter, we looked into the influence of Col17 expression on keratinocytes. It has already been shown that Col17 deficient keratinocytes present a different phenotype *in vitro* in comparison to wild-type keratinocytes. First, the Col17 negative cells were found to have accelerated, but less directed, motility when compared to wild-type keratinocytes.[25,26] The recent study by Löffek et al. also shows that the migratory phenotype of Col17 negative cells is associated with enhanced phosphoinositide 3-kinase (PI3K) activity.[25] In 2006 Pankow et al. showed that PI3K is an important regulator of epidermal homeostasis and wound repair and that stimulation of Pl3K activity in cultured keratinocytes promoted cell proliferation.[27] Downstream from the Pl3K we find the NF-κB pathway. Van den Bergh et al. showed that Col17 deficiency causes a proinflammatory reaction in cultured keratinocytes driven by the NF-κB pathway and that Col17 deficient keratinocytes had higher levels of the NF-κB reporter than wild-type cells, suggesting higher stimulation of this pathway.[28] Not surprisingly, NF-κB is also found to be involved in epidermal homeostasis.[29] One could thus hypothesize that higher activity of Pl3K and NF-κB pathways in Col17 deficient cells than in the wild-type or revertant keratinocytes might be the reason for faster proliferation of Col17 mutant cells when cultured *in vitro*. Recently, during the 2014 congress of the European Society for Dermatological Research, Marsh et al. showed that siRNA Col17 knockdown keratinocytes show higher proliferation rates than wild-type cells giving an important argument supporting our theory.[30] Further investigation of revertant and mutant cells and their proliferation rate is needed, but in the future NF-κB inhibitors like Bay-11-7082,[28] might help to protect revertant keratinocytes during *in vitro* expansion from being overgrown by mutant keratinocytes. In **Chapter 6** we have presented our efforts to enrich the *in vitro* cell culture with Col17 revertant cells.

In 2007, in the study that aimed to asses the retroviral insertion safety for gene therapy of skin diseases, Larcher et al. was able to isolate a single holoclone and expand it *in vitro* to amounts needed for culture of skin equivalents.[33] Selection of a single Col17 revertant stem cell, a holoclone, could therefore lead to sufficient *in vitro* expansion to produce enough skin grafts to cover the whole human body. If possible, instead of one holoclone, more revertant cells with high proliferative potential could be selected by dilution of keratinocytes mass cultures combined with colony forming efficiency assay, as described by Larcher et al., to achieve not a monoclonal but a polyclonal revertant culture.[33] From personal communication with F. Larcher, we know that such selection is difficult and has not yet been achieved for revertant keratinocytes *(unpublished data)*.

Research on epidermal stem cells is evolving rapidly and, as described in **Chapter 1**, multiple keratinocyte stem cell markers have been already proposed. Visualisation and possible selection of revertant stem cells could help to establish a pure revertant cell culture, which would then allow production of fully revertant skin grafts. One of the options to show the number of revertant stem cells and their localisation would be to use biopsies from revertant skin and stain them with a stem cell marker. We have tested several monoclonal antibodies that are described in literature to stain epidermal stem cells. Worth mentioning are: DF1513 (CD71), GOH3 (integrin alpha 6),[34] A11b2 (integrin beta 1),[35] LHK15 and SPM190 (both keratin 15), 4A4 (p63)[36] and nb500 (survivin)[37] antibody. When stainings of normal human skin were analysed we discovered that there was no overlap between cell populations stained with those markers. It is possible that each of the antibodies visualized a stem cell rich population, however we were unable to pinpoint the revertant stem cells with immunofluorescence only. Further studies with new markers combined with colony forming efficiency assays and flow cytometry could help to establish an estimate of the revertant stem cell population. This will definitely add more to the understanding of revertant mosaicism as a phenomenon and in the more distant future could be translated to improve revertant cell therapy.

Other groups are also working on the development of revertant cell therapy. A clinical trial aimed at transplantation of cultured revertant skin grafts was registered by Stanford University on the www.clinicaltrials.gov between October 2011 and April 2014. It was, however, withdrawn prior to enrolment of the subjects and no data from this study are openly available. We hope that this thesis will encourage others to look for possible solutions to the problem of *in vitro* expansion of Col17 revertant cells. Ultimately, we believe that after future research, safe and successful selection techniques for Col17 revertant cells will be developed. In **Chapter 6** we showed an unsuccessful attempt to expand Col7 revertant cells because of the low amount of naturally corrected keratinocytes in the original biopsy. Such simple *in vitro* expansion of revertant keratinocytes isolated from patients with lam-332 or Col7 deficiency should be further studied, as there is currently no rationale to expect *in vitro* growth advantage of mutant cells.

REVERTANT CELL THERAPY: A NEW CONCEPT

Inducing pluripotent stem cells from revertant keratinocytes

Presuming that revertant skin grafts can be produced, *in vitro* expansion levels are often limited, for example by patient's age. Moreover, extracutaneous manifestations of EB, cannot be treated with transplantation of cultured epidermal grafts. To take full advantage of the presence of revertant keratinocytes, a combination of present knowledge with new developments in regenerative medicine is needed. The technique of inducing pluripotent stem cells (iPSCs) from a somatic cell, for which the Nobel Prize in Medicine in 2012 was awarded to John B. Gurdon and Shinya Yamanaka, can contribute to broaden the application of revertant mosaicism in therapy of all genetic diseases.[38-40] Cells with properties of an embryonic cell can be obtained from an already differentiated somatic cell by introduction of selected transcription factors (OCT4, c-MYC, KLF4 and SOX2).[39] IPSCs have been induced from skin fibroblasts, bone marrow derived mesenchymal cells and epidermal keratinocytes.[38,40-42] Induction of iPSCs from revertant tissue leads to patient specific, naturally corrected cells with theoretically unlimited expansion potential – a perfect tool for treatment of genetic diseases. Induction of pluripotent stem cells is at the moment widely performed with retroviral vectors. This is a very effective method, however safety concerns arise, as usage of viral vectors may be carcinogenic. Moreover, it is of importance to successfully differentiate IPSCs to target lineages to prevent teratoma formation. In order to find more clinically friendly approaches for iPSCs induction, the usage of transcription factors in the form of recombinant proteins, integration-free viral vectors or plasmid-based derivation could be applied.[43-45] Noteworthy is a very elegant strategy of synthetic mRNA introduction, which does not require integration within the genome and can be easily controlled owing to the quick degradation of mRNA.[46]

Recently iPSCs have been acquired from revertant skin of patients with EB. Tolar et al. isolated revertant keratinocytes from a healthy skin patch of a 10-year-old boy with severe generalized RDEB due to two loss-of-function mutations in *COL7A1*, paternal c. 3840delC and maternal g.6751-2A>G.[42] Keratinocytes isolated from the revertant patch showed the presence of the paternal mutation and, due to the skipping of exon 86, the maternal mutation was not present. This resulted in a shorter, but presumably functional *COL7A1* transcript. By using retroviral vectors, keratinocytes were reprogrammed into iPSCs, which then were differentiated into haematopoietic progenitor cells and keratinocytes. Our recent cooperation with Dr. Christiano's group from University of Columbia resulted in the induction of iPSCs from revertant keratinocytes isolated from a healthy skin patch of JEB-gen-intermed due to mutations in *COL17A1* gene.[47] This is the same patient that was described in 1997 by Jonkman et al. and is also involved in the research described in **Chapters 2 and 3** of this thesis. Revertant keratinocytes and therefore also iPSCs showed only the paternal mutation, while the maternal mutation was corrected due to

gene conversion. These cells were expanded *in vitro* and differentiated into keratinocytes, which could then form not only a 3D skin equivalent expressing wild type Col17, but also full thickness human skin in a murine model. Both studies were performed with introduction of retroviral vectors with required transcription factors. The next step would be to use more clinically applicable methods of iPSCs induction followed by *in vivo* testing in animal models, which would bring us closer to cultured revertant grafts obtained through induced pluripotent stem cells.

Systemic therapy with revertant cells – the ultimate goal

In **Chapter 1** we reviewed the usage of allogeneic stem cell transplantation for treatment of RDEB described in 2010.[48] Recently, a clinical study that uses transplantation of umbilical cord blood instead of bone marrow as a haematopoietic stem cell source combined with mesenchymal stromal cells following reduced intensity conditioning has started in the Netherlands and aims to achieve at least the same levels of disease amelioration, as achieved in the Minnesota trial, combined with improved safety and reduced mortality. Both Minnesota and Dutch studies are based on the fact that allogeneic stem cells that can express Col7, consist not only of haematopoietic stem cells but also of the Lin–/PDGFRα+ population that can transform to epidermal and dermal cells.[49] Tamai et al. not only characterized the Lin–/PDGFRα+ population, but also identified the SOS signal that mobilizes this population to regenerate injured epithelium. High mobility group box 1 protein (HMGB1) is highly expressed in patients with RDEB due to extensive blistering and therefore the presence of hypoxic keratinocytes. It is in response to these keratinocytes that HMGB1 can thus mobilize the Lin–/PDGFRα+ subpopulation of transplanted allogeneic stem cells to become Col7 expressing epidermal cells.[49] When Tolar et al. induced IPSCs from revertant keratinocytes; they also successfully differentiated them into the haematopoietic stem cells.[42] One could therefore imagine the autologous, naturally corrected haematopoietic stem cells containing Lin–/PDGFRα+ cells derived from revertant keratinocytes through induction of iPSCs and their subsequent auto-transplantation. Although such a "from skin to blood to skin approach" would still require certain conditioning prior to transplantation in order to create a niche, no graft versus host disease prophylaxis would be required. Revertant pluripotent cells could be expanded for this *in vitro* and differentiated into haematopoietic cells, mesenchymal and epidermal stem cells. This means that if needed, skin grafts could be produced to cover the whole body area of an EB patient or that systemic application of revertant haematopoietic stem cells would be possible.

With the induction of revertant iPSCs broadening the possible application of revertant cell therapy, we should not forget that this requires temporary genetic manipulation and therefore removes the earlier mentioned advantage of revertant cell therapy being free of artificial changes in the genome.

Translation of revertant cell therapy in EB to other genetic diseases

Revertant cell therapy in EB has received a lot of attention and interest. We believe that skin diseases will continue to be on the forefront of revertant cell therapy due to relatively easy measurement methods to monitor success of such therapy and the unique visual outcomes. Induction of iPSCs from revertant cells may also be used in other genetic diseases, such as WAS. One could easily speculate about iPSCs induced from revertant lymphocytes and transplantation of autologous haematopoietic stem cells in WAS. Currently therapy of WAS consists of protein replacement therapy, allogeneic stem cell transplantation or gene therapy, and therefore transplantation of autologous naturally corrected stem cells would remove the need for persistent change in genome of transplanted cells or graft versus host prophylaxis.[50] Not forgetting this theoretical advantage, it is too early to speculate if revertant cell therapy for WAS would be superior to therapies already being applied.

Revertant cell therapy: important element of personalized medicine for EB

In **Chapter 1** we discussed different approaches to treat EB. Recently, a newsletter issued by the EB patients organisation DEBRA reported that at Stanford University, USA, the first patient with RDEB was treated with transplantation of autologous skin grafts made from cells corrected by gene therapy.[51] Additionally, a clinical trial started in Salzburg, Austria for gene therapy in patients with JEB-gen-intermed has been reported in the same newsletter. The first RDEB patient has been enrolled in the aforementioned clinical trial of umbilical cord blood transplantation for EB in the Netherlands. As the many different therapies for EB mentioned in this thesis are becoming applicable, a personalized medicine approach to treat EB can be proposed (Figure 1). We believe that in the future, therapy for each EB patient will be considered separately after assessment of key factors: subtype of EB, type of mutation, age, extent of cutaneous and extracutaneous symptoms and presence of revertant mosaicism.

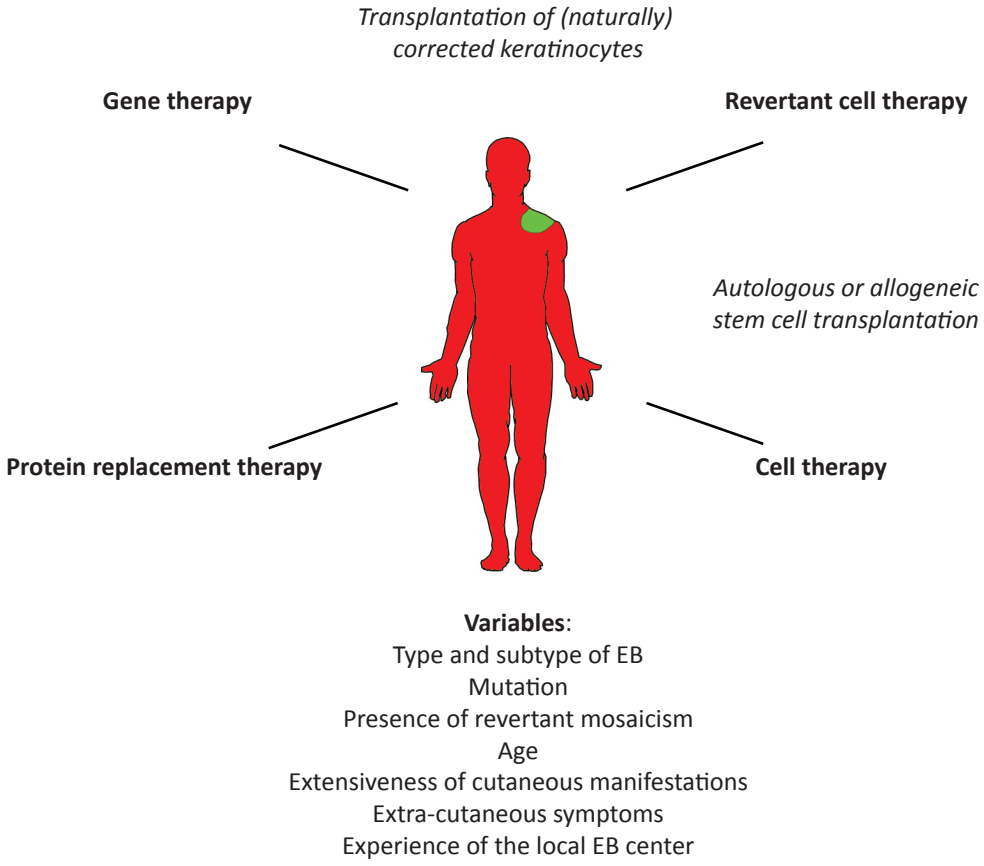

Figure 1. Schematic presentation of a future, personalized model of EB treatment with four major therapeutic approaches for EB in **bold** and with overlap between revertant cell therapy and cell therapy and gene therapy in *italic*.

REVERTANT *MOSAICISM*: A GOLD MINE FOR RESEARCH AND CLINICS

Discussion over perspectives for revertant cell therapy show the importance of the phenomenon of revertant mosaicism to the clinics. In addition to that, **Chapter 5** of this thesis shows how patients with revertant mosaicism can contribute to research on human biology.[52] In **Chapter 5**, we used reverse translation, from bedside to the bench, to analyse the hyperpigmentation of revertant patches in Col17 deficient patients. Although Tanimura et al. have already suggested the influence of Col17 on melanocytes in the murine model,[53] prior to the study described in this thesis no proof of such a fact had been shown in humans. Our study showed a significant correlation between the presence of melanocytes, pigmentation and Col17 expression. The model suggested by Tanimura, where paracrine stimulation of melanocyte stem cells by epidermal stem cells is missing when the latter expresses no Col17, may explain the dependence between Col17 deficiency and lack of melanocytes.[53] There are, however, other mechanisms that involve Col17, for example an increased proinflammatory response due to Col17 deficiency, as the earlier discussed study by Van den Bergh et al..[28] showed. Increased stimulation of interleukins 6 and 8 can lead to targeting of melanocytes by the immune system and inhibition of their growth.[54-56] As more studies are needed to further clarify these theories on reasons for melanocyte dependence on Col17 and possibly Lam-332, as described in **Chapter 5**, our observations and conclusions show that revertant mosaicism can be an important tool not only to develop successful therapies for EB, but also to perform research on this extremely interesting population of human knock-outs.

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