Revertant cell therapy for epidermolysis bullosa

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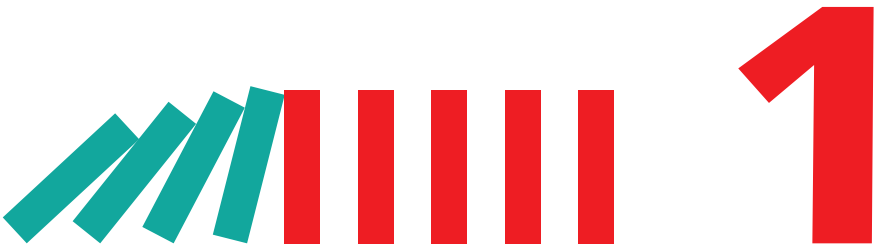

INTRODUCTION

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This thesis describes a quest to understand and use the phenomenon of revertant mosaicism, also known as 'natural gene therapy', to treat the heritable skin disease, epidermolysis bullosa (EB). This introduction chapter can be divided in three parts. We will start with a brief description of the human skin as an organ, followed by details about its two most important cell populations, keratinocytes and fibroblasts. Keratinocyte stem cell biology will be presented in regard to its current clinical applications. Next, a characterization of the dermal-epidermal junction will follow that leads to the description of EB, i.e. the disease caused by mutations affecting proteins involved in epidermal adhesion. Current research on the therapeutic approaches for EB will be briefly presented, which will later allow us to discuss the place for revertant cell therapy. Finally, in the last part the concept of revertant cell therapy will be formulated and at the end of this chapter the aims and an outline of this thesis will be presented.

1

THE HUMAN SKIN

The skin is the largest organ in the human body, weighing approximately 5 kg with an area of 1.5-2 m^2 in adults.[1,2] It forms, together with its appendages, the integumentary system. This system prevents penetration of UV radiation, allergens, toxic substances and other organisms, while also protecting the body from water loss and trauma.[3] The skin also takes part in immune reactions, sensory perception, vitamin synthesis, temperature regulation and secretion of waste. It is also important to mention that the skin plays a role in the social and sexual interaction and communication between human beings.[4]

The skin is composed of two compartments with the epidermis being the outer compartment, and the dermis the underlying connective tissue compartment. These two compartments are connected by the complicated system of adhesive proteins interacting with each other in the basement membrane zone[3] (Figure 1).

Epidermis

The epidermis is a continuously renewing stratified squamous epithelium derived from the ectoderm and averages 50 µm in thickness. Keratinocytes are the major cell population building the epidermis (about 90%) and are organized in four layers representing different stages of differentiation (Figure 1), while on the palms and soles a fifth layer, stratum lucidum, can be found. Aside from keratinocytes, in the epidermis we can find melanocytes, Langerhans cells and Merkel cells.[3] As knowledge about keratinocytes and melanocytes is important for this thesis, those two cell populations will be discussed beneath in more detail.

Keratinocytes

Keratinocytes are named after the keratin intermediate filament protein they contain and are the majority of the cells found in the epidermis. Keratinocytes connect with each other via desmosomes and tight junctions, which provide attachment sites for keratin filaments, present in the cytoplasm and participate in signal transduction and differentiation. The cytoplasm of neighbouring keratinocytes is also channeled by gap junctions, that allow ion and low molecular metabolite exchange.[5] The turnover time of the epidermis, ie. time needed for a keratinocyte to differentiate and migrate from the basal layer to stratum corneum, is about four weeks.[6] The constant renewal, and regeneration following trauma of the epidermis, can be achieved because a population of self renewing epidermal stem cells is present in the basal cell layer.[7]

Epidermal stem cells

Traditionally it has been believed that epidermal stem cells are multipotent, clonogenic and slow cycling. Through asymmetric division they keep their stem cell abilities while giving rise to a

rapidly-cycling transient amplifying cell (TA).[8] In this theory, the stem-cell itself divides rarely, but the TA cell feeds the surrounding differentiated keratinocyte population of new cells ("amplifying"), before final differentiating after a limited number of divisions ("transient").[8,9] There are, however, opposing opinions promoting the theory that a single, constantly cycling progenitor cell gives rise to all the surrounding basal keratinocytes without the TA cells, while maintaining its ability to undergo an unlimited number of divisions.[10] While discussion on the fate of epidermal stem cells is maintained, many groups aim to identify a specific epidermal stem cell marker. At the moment murine epidermal stem cells are believed to express the following surface proteins: Lgr5, Lgr6, CD34, Plet1, Lrig1;[11-15] while also having a high expression of the transcription factor, p63.[16] However, it is still unclear how much of this knowledge can be translated into the understanding of the human epidermis.[8] Expression levels of proteins, such as CD71[17], α6 integrin[18], β4 integrin[19], Lrig1[20], ABCG2[21] or p63[16] can help to identify stem cell rich cell populations. Moreover, high expression of keratins 15 and 19 has also been described in epidermal stem cells.[22] Unfortunately, all these proteins fail to pinpoint a single stem cell and thus cannot be used as a stem cell specific marker.[23]

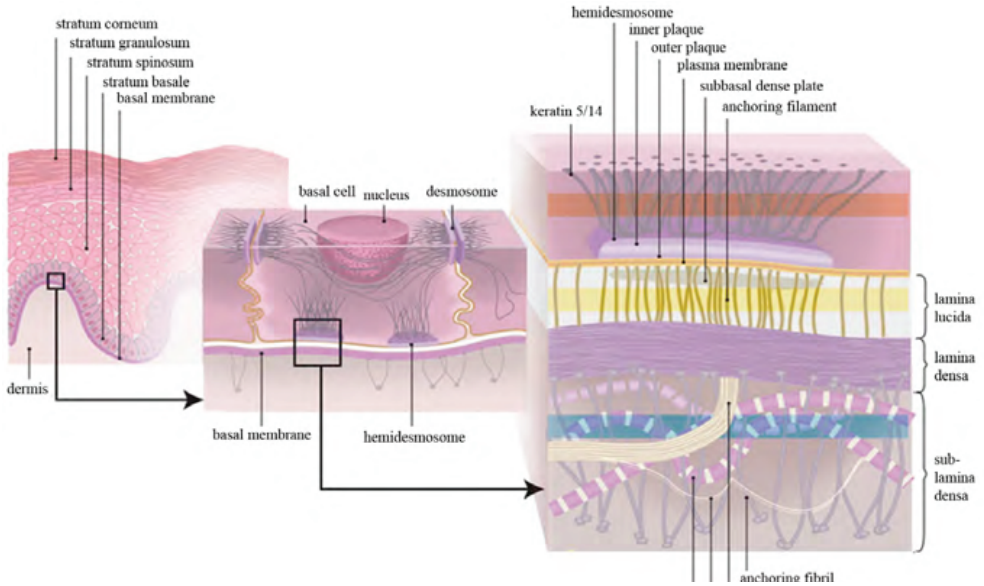


Figure 1 To the left the organisation of epidermis in four layers and basal membrane connecting it to the underlying dermis. In the center we see the magnification of the dermal epidermal junction with the details of the basal membrane zone and hemidesmosome adhesion complex to the right. Courtesy of Prof. dr. M.F. Jonkman[73]

Epidermal cells in regenerative medicine

The history of skin transplantation began in ancient India, where autologous soft tissue flaps were used to cover nasal and ear defects.[24] In 1869 a Swiss surgeon, Jacques-Louis Reverdin, performed the first autologous skin grafting procedure. Since then, autologous grafting of the skin has become widespread and currently the split-skin and full-thickness grafts are a standard procedure.[24] These methods use patients' own skin, which is harvested and then placed on the wound bed, the acceptor site. Autologous skin transplantation can cover areas slightly larger than the donor site, while meshed grafts allow coverage of areas theoretically up to 9 times larger that the donor site but 2-4 fold expansion is more realistic. Unfortunately even such expansion ratios can be insufficient in cases of extensive burn wounds and thus limit donor site areas. The revolutionary work of Rheinwald and Green on culturing human epidermal cells into sheets suitable for grafting resulted in the first transplantation of a cultured keratinocyte graft described in 1981 by O'Connor et al.[25]. Techniques for autologous cultured epithelial autografts were later refined but the technique developed by Rheinwald and Green, involving a feeder layer of murine fibroblasts, still serves as the gold standard.[23] Currently cultured autologous skin grafts are used in treatment of chronic wounds and burns in plastic and reconstructive surgery while also being proposed as the vehicle for genetically modified keratinocytes in gene therapy for genodermatoses.[23,26] Although cultured skin grafts provide the highest expansion ratio, it takes about 3-4 weeks for grafts to be fully grown.[27] This is why other techniques of transplantation of epidermal stem cells, such as enzymatic isolation of epidermal cells and transplantation in the form of cell suspension, are being used for the treatment of chronic wounds, burns and vitiligo.[28-30]

Melanocytes

Melanocytes are derived from the neural crest and can be found in the basal layer of the interfollicular epidermis, in the ratio of 1:10 with keratinocytes, and in the bulb of the hair follicle, where the ratio is lower, 1:5.[31,32] Melanocytes are responsible for production of melanin and transport of melanosomes; organelles containing melanin, to surrounding keratinocytes.[33] Furthermore, the crosstalk between keratinocytes, fibroblasts and melanocytes seem to have an influence on skin homeostasis.[33] Melanocyte stem cells are believed to be in the bulge of the hair follicle, whereas a biologically different melanocyte stem population seems to be present in the dermis.[33] Epidermal stem cells interact with melanocyte stem cells. This will be further discussed in **Chapter 5** and **6** of this thesis. The quest for identification of the melanocyte stem cell is, however, unfinished since a specific human melanocyte stem cell marker has not been discovered yet.

Dermis

The dermis is built from a cellular component, consisting of fibroblasts, macrophages, dendritic cells and mast cells surrounded by the extracellular matrix composed of collagen and elastic fibres, enmeshed by an extrafibrillar ground substance consisting of proteoglycans. Fibroblasts are mesenchymally derived and are the major cell type of the dermis. They are responsible for synthesis of the extracellular matrix. Furthermore, nerves and blood vessels can also be found in the dermis allowing it not only to function as structural support for the epidermis, but also for provision of nutrition.[3]

Basement membrane zone

The basement membrane zone (BMZ) is a sheet-like structure that separates the dermis from the epidermis and is the site of adhesion between these two compartments.[34] The BMZ is a dynamic interface that regulates proliferation, adhesion, differentiation, migration and apoptosis.[35,36] Structures responsible for anchorage of basal keratinocytes to the dermal extracellular matrix are the hemidesmosomes and the focal adhesion complexes[35] (Figure 2). The hemidesmosome complex starts in the basal keratinocytes, where keratin filaments composed of keratin 5 and 14 connect with the inner plaque formed by BP230[37] and plectin.[38,39] It is then connected to the outer plaque, which is built from cytoplasmic domains of type XVII collagen[40] (Col17, also known as BP180) and α6β4 integrin (α6β4).[41] CD151[42] together with the extracellular domains of Col17 and α6β4 forms an extracellular sub-basal plate running parallel to the keratinocyte membrane, while Col17 and α6β4 also connect with laminin-332 (lam-332). Lam-332 together with Col17, laminin-311 and laminin-511 form anchoring filaments, which cross the lamina lucida from the sub-basal dense plate to lamina densa.[38,43] The lamina densa is an electron dense layer that consists mainly of type IV collagen together with nidogen, perlecan and other glycoproteins.[44] The anchoring filaments bind within the lamina densa through the β3 laminin chain of lam-332 to anchoring fibrils (AFs) build from type VII collagen (Col7) that are semicircular structures that extend from the lamina densa to the dermis and then loop back, securing adhesion to the extracellular matrix of the dermis.[45] The focal adhesion complex binds the actin skeleton to the extracellular matrix of the dermis. It starts with the actin microfilament skeleton that binds integrins through kindlin-1. The most important integrin involved in the focal adhesion complex is α3β1 integrin. The extracellular domain of the integrins connect to the laminins, mostly lam-332, which mediates its adhesion to the extracellular matrix of the dermis.[35,46]

Mutations in genes encoding proteins of hemidesmosome and focal adhesion complexes cause epidermolysis bullosa (EB), a group of inherited diseases. In this thesis we will focus on subtypes of EB caused by mutations in genes coding for the Col17, lam-332 and Col7 proteins.

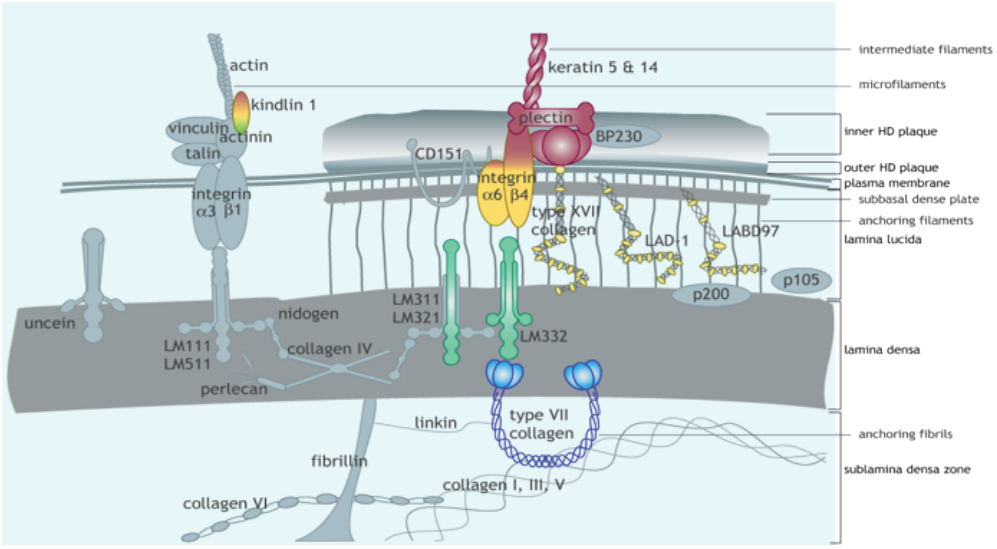


Figure 2 Overview of the hemidesmosome adhesion complex (right) and focal adhesion complex (left) with proteins associated with epidermolysis bullosa in colour. Courtesy of Prof. dr. M.F. Jonkman

Type XVII collagen

Encoded by the gene *COL17A1* that spans 52 kb on chromosome 10q24.3 and consists of 56 exons, the Col17 protein has a molecular mass of 180 kD.[47,48] The *COL17A1* gene is expressed in cornea, teeth, mucous membranes, brain, placenta, umbilical cord and in the skin. It is important to add that in the skin only basal keratinocytes express *COL17A1*.[40] Col17 is a homotrimer built from α1 (XVII) collagen chains of 1,497 residues and has a type II orientation, meaning that the N-terminus (466 residues) starts intracellularly, while the C-terminus (1008 residues) ends extracellularly. The flexible collagen tail domain extends up to the lamina densa.[49] The intracellular domain of Col17 binds to β4 integrin, plectin and BP230, while the extracellular domains binding partner is lam-332.[50,51] Aside from the very important role in dermal-epidermal adhesion, Col17 is important for cell migration and signal transduction.[52-54] Col17 can also be a target for autoantibodies thereby leading to cutaneous pemphigoid, pemphigoid gestationis, lichen planus pemphigoides, linear IgA disease, cicatricial pemphigoid and mucous membrane pemphigoid.[55] Deficiency of Col17 leads to generalized intermediate junctional EB (JEB-gen intermed), which will be discussed later in this chapter.[56]

Laminin-332

All laminins consist of three different laminin polypeptides α, β and γ. Until now, 16 different laminins have been reported, and 5 α, 3 β and 3 γ chains have been identified. Lam-332 is a heterotrimer composed of α3, β3 and γ2 chains encoded by *LAMA3*, *LAMB3* and *LAMC2* respectively.[57] In the epidermis, it is mainly synthesized and secreted by keratinocytes, but actively dividing melanocytes can also secrete lam-332.[58] The assembly of lam-332 starts with the formation of a β3 and γ2 dimer, while the α3 is incorporated later in the endoplasmic reticulum and lam-332 is secreted extracellularly as a heterodimer precursor.[59] Before lam-332 can become a part of the hemidesmosome adhesion complex, it undergoes extracellular proteolytic processing that shortens the 200 kDa α3 chain to 145 kDa and the 155 kDa γ2 chain to 105 kDa, while the 140 kDa β3 chain remains intact.[60] From the epidermal side of the dermal-epidermal junction, lam-332 is connected to α6β4 and Col17, while on the dermal side it connects to Col7 in AFs. Lam-332 is a crucial component of the hemidesmosome adhesion complex.[61,62] Lam-332 is also important for cell migration, in normal epithelial cells, while in invading malignant epithelial cells lam-332 is often overexpressed.[63] The role of lam-332 expression in actively dividing melanocytes has not yet been discovered.[58]

Similar to Col17, autoantibodies can also target lam-332 causing mucous membrane pemphigoid and cicatricial pemphigoid. Lam-332 deficiency causes JEB with the severity depending on the mutation type: nonsense mutations on both alleles encoding one of the chains results in severe generalized junctional EB (JEB-gen sev) that is often lethal,[64,65] whereas presence of at least one missense or splice-site mutation results in non-lethal severe intermediate junctional EB (JEB-gen intermed).[65]

Type VII collagen

The *COL7A1* gene is localized on chromosome 3, p21.31 and consists of 118 exons. Despite the fact that the number of exons is large, the *COL7A1* gene consists of only 31088 bp, and through a cDNA of 8832 bp gives rise to the pro-α1 (VII) peptide, a 2944 amino acid precursor of Col7.[66,67] Pro-α1 (VII) consists of the N-terminal non-collagenous 1 (NC1) domain, triple helix domain in the middle and the C-terminal non-collagenous 2 (NC2) domain. The triple helix domain consists of Gly-X-Y repeats characteristic for collagens, which are interrupted by non-collagenous sequences.[68]

The pro-α1 (VII) peptide undergoes post-translational modifications and three pro-α1 (VII) peptides form a homotrimer, which is secreted to the extracellular matrix. The NC2 domains of the homotrimer are then cleaved by bone morphogenetic protein-1 in exon 115, which allows two homotrimers to dimerize and form AFs with NC1 domains pointing outwards. The NC1 domain contains regions that allow AFs to bind to the lam-332 and integrins, and is thus responsible for

the adhesion of the AFs to the anchoring filaments.[69] Col7 in the skin is synthesized mainly by keratinocytes and, in smaller quantities, by dermal fibroblasts. Col7 is also present in mucosal and bronchial epithelium.[70] Mutations in *COL7A1* lead to different dystrophic subtypes of EB (DEB).[69]

EPIDERMOLYSIS BULLOSA

EB is a group of heterogenic genodermatoses that share blistering of the skin after minor trauma as a symptom. A recent consensus on classification reports that at least 18 genes coding for epi- dermal and dermal proteins (Figure 3) are known to be responsible for causing EB.[65] The level of blister formation in the skin divides EB into four major types but more than 30 subtypes were distinguished based on the affected protein, disease severity, distribution of lesions and presence of extracutaneous manifestations.[65] The annual incidence of EB is about 1 per 17,000 births.[71,72,73]

The severity of clinical manifestations of EB varies from mild to lethal. EB is thus characterized by huge phenotypic variability, depending on the affected gene, type of the mutation, consequence on mRNA, posttranslational modification of expressed protein, and expression level. Apart from cutaneous manifestations, EB can affect gastrointestinal, genitourinary and respiratory tracts; cause growth retardation, anaemia, pseudosyndactyly and increase the risk of developing skin malignancies.[65] Some mutations lead to death within the first few months after birth. According to the newest consensus, the diagnostic process of EB involves identification of the level of cleavage with immunofluorescence (IF) antigen mapping and/or transmission electron microscopy on fresh blisters. Furthermore, immunofluorescence staining for specific antigens is used to identify the affected protein, assess expression level and therefore help to determine the subclassification. When the candidate protein is identified, mutation analysis is performed and the candidate gene is sequenced to allow final subclassification and help with genetic counselling.[65] IF and mutation analysis are also very important in the identification of revertant mosaicism, which will be the subject of the next part of this introduction and will lead directly to the aims of this thesis. However, before we move forward, two major subtypes of EB, junctional and dystrophic EB, will be described in more detail.

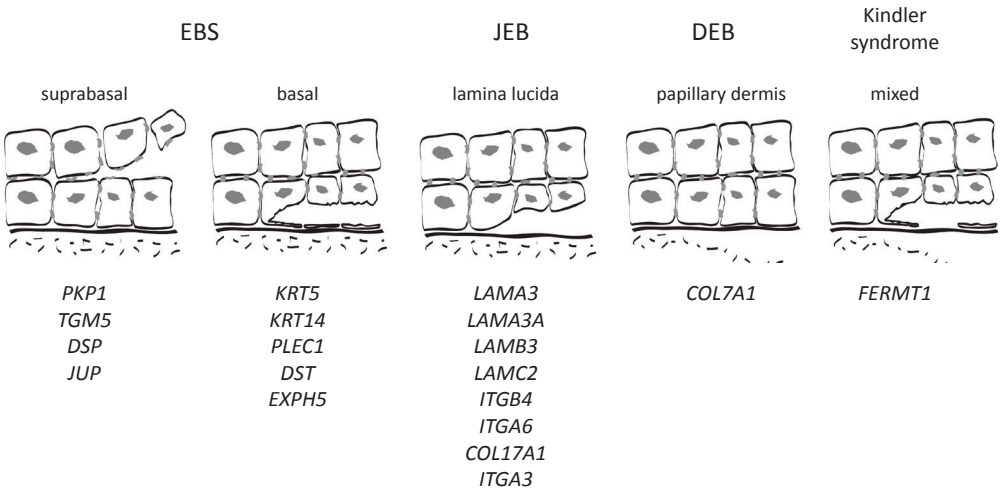


Figure 3 Graphic representation of the level of blister formation in different subtypes of epidermolysis bullosa: EB simplex (EBS), junctional EB (JEB), dystrophic EB (DEB) and Kindler syndrome. Below the genes involved in the corresponding subtype are listed. Courtesy of Prof. dr. M.F. Jonkman

Junctional epidermolysis bullosa

JEB is caused by mutations in genes encoding the following proteins Col17 (*COL17A1*),[74] lam-332 (*LAMA3*, *LAMB3* or *LAMC2*)[75-77] or integrins, α6β4 (*ITGA6* and *ITGB6*) and α3 subunit (*ITGA3*). Clinical phenotypes are for the large majority inherited autosomal recessively.[65] In JEB, blistering occurs within the lamina lucida of the BMZ. According to the recently published classification, JEB is divided in a generalized type (JEB-gen), with widespread blistering and a localized type (JEB-loc) with milder blistering, often only on hands and feet, and not affecting hair growth. Within the JEB-gen subtype, the form caused by total absence of functional lam-332 earlier named JEB-H is now named JEB-generalized severe (JEB-gen sev).[65] When a child with this form of EB is born, the blistering of the skin and mucous membranes results in complications such as anemia, dyspnoea and failure to thrive, which are so severe that they lead to death with an average life expectancy of 6 months.[64] The other main form of JEB earlier named JEB-nH can be caused by reduced levels of lam-332 or absent levels of Col17 and causes severe blistering and is now named JEB-generalized intermediate (JEB-gen intermed). Patients with JEB-gen-intermed and mutations in lam-332 have an increased risk of developing cutaneous squamous cell carcinoma (SCC).[78]

Dystrophic epidermolysis bullosa

All subtypes of DEB are caused by mutations in the *COL7A1* gene, which leads to defective and/or reduced numbers of AFs.[79] DEB can be inherited both autosomal recessively and dominantly. Blistering in DEB occurs below the BMZ, in the papillary dermis, which in contrast to other forms of EB, can lead to scarring. Severity of recessive DEB (RDEB) depends on the mutations combinations, which can result in different amounts of Col7 expression.[80] Disease subtype correlates with the amount of Col7 present in the dermal epidermal junction when determined by immunofluorescence.[80]

Patients with RDEB experience spontaneous blistering after the slightest mechanical stimuli and heal with extensive scarring resulting in fibrotic scarring, atrophy of the skin and often loss of hair follicles. Of all patients wtih EB, RDEB generalized severe (RDEB-gen-sev) patients experience the poorest quality of life. Characteristics symptoms of RDEB-gen-sev are pseudosyndactyly, a gradual fusion of fingers and toes, leading to deformation of hands and feet, microstomia, dysphagia, anemia and growth retardation. Long-term complications of RDEB-gen-sev are renal failure and development of highly aggressive SCC, which results in death of the majority of patients before 40 years of age.[81]

THERAPEUTIC APPROACHES FOR EB

At the moment there is no cure for EB.[82] There are therapies that have great potential to become one in the future and there are therapies that ameliorate the course of the disease. This thesis focuses on the quest to establish a therapeutic approach using the phenomenon of revertant mosaicism in EB. As revertant mosaicism and the concept of the revertant cell therapy will be discussed in the next part of the introduction, it is of importance to first introduce strategies that are currently under investigation to cure EB.

When discussing therapy the therapy for a genetic disease, three main groups can be identified:[83-85]

- Gene therapy wich aims to introduce a wild-type copy of a gene into the patient's body, which will result in a wild type phenotype. It can be achieved *ex-vivo/in-vitro* or *in-vivo*.
- Protein therapy, which aims in supplementation of the missing or non-functional protein with a protein obtained from healthy donors or a recombinant one.
- Cell therapy, which encompasses therapeutic approaches using allogeneic or autologous cells to directly or indirectly reinstate the missing or non-functional protein.

All three above mentioned strategies are being developed for EB. We will briefly describe the idea behind each strategy, while the advantages and disadvantages of each approach will be described and debated in the discussion chapter **(Chapter 6)** of this thesis.

Gene therapy for EB

Gene therapy for EB aims to correct the affected gene in the epidermal cells or introduce the wild-type copy into the keratinocytes. The best illustration of how this approach works, is the only published successful case of gene therapy in a patient with EB. In 2006 Mavillo et al. reported the successful treatment of a JEB-gen-intermed patient with mutations in *LAMB3*.[26] Using a murine leukemia virus based retroviral vector expressing long terminal repeat driven *LAMB3* cDNA, patient's keratinocytes containing epidermal stem cells, earlier isolated from a skin biopsy, were tranduced *ex-vivo* and transgenic epidermal grafts were cultured *in vitro*. Affected epidermis on the patient's upper legs was removed surgically and cultured grafts were placed on the wounds. In 2014 a follow-up report showing stable expression of lam-332 in the transplanted regions after 6.5 years (80 complete renewing cycles of the transplanted epidermis) was published.[86] Further clinical trials were, however, stopped due to safety concerns by the regulatory committees, as possibilities of random genomic integration of the viral vectors and its consequences deemed to be better understood.[87] Since 2006, many more viral vectors, not only for *LAMB3* but also for other EB genes, for example *COL7A1* were introduced and usage of viral vectors became very efficient to correct the defect in patient cells.[88,89] More data about safety of viral vectors have been acquired and clinical trials will follow shortly.[87] Recently however, techniques allowing in situ correction of the genome using genome-editing strategies, as zinc-finger nucleases (ZFNs) or transcription activator like effector nuleases (TALENs), have been introduced to remove the risk of insertional mutagenesis.[90] TALENs for example have been used successfully to correct *COL7A1* in human fibroblasts *in vitro,*[91] but this approach is still far from clinical trials.

Protein therapy

Substitution of the missing or non-functional protein in genetic disorders of enzyme production has been already used in situations, where the affected cells can take up the enzyme. Recent studies showed that employment of protein therapy in cases of defective structural protein could also lead to promising results.[92,93] Intradermal injections of recombinant Col7 resulted in the incorporation of Col7 in wounded skin, but not in internal organs and unaffected skin.[94,95] As intradermal injections can be painful and, even if Col7 is a very stable protein, would have to be repeated a better systemic approach is needed. Col7 is a soluble protein, thus systemic, intravenous application is feasible. Woodley et al. recently reported restoration of anchoring fibril formation and dermal-epidermal adherence in a murine model of RDEB by intravenous injection of recombinant Col7.[96] Although this approach seems very close to clinical translation, other groups have raised safety concerns regarding the possible immunological response to recombinant Col7.[87,97]

Cell therapy

Autologous skin grafting has been used for many years to close wounds in patients with EB.[98-100] In the last decade dispersions of allogeneic and autologous cells were used *in situ* or systemically. Examples are, intradermal injections with allogeneic fibroblasts or with mesenchymal stromal cells, and systemic hematopoietic stem cell transplantation.[83,85,87] Wong et al. showed in 2008 that intradermal injection of allogeneic fibroblasts in RDEB can upregulate *COL7A1* expression for 3-6 months.[101] A study comparing a single intradermal set of injections with allogeneic fibroblasts in and around a skin erosion showed greater reduction of wound area up to day 28 compared to only vehicle injection, but the difference seemed to be gone at the 6 months timepoint.[102] This study also failed to show statistical significance between the fibroblast and the vehicle group, probably due to small number of treated lesions. The compared group showed no difference between fibroblasts and vehicle injections with both significantly accelerating the wound healing process and similar expression of Col7.[103] These results suggest that fibroblasts themselves may not be crucial for the positive effect on wound healing but the upregulation of *COL7A1* by the heparin-binding epidermal growth factor-like growth factor (HBEGF) could be the mechanism of action.[102,103]

In 2007 a study with bone marrow transplantation for RDEB began in Minnesota, USA and was published in 2010.[85,104] Children with RDEB received myelo-ablative chemotherapy and transplantation of allogenic stem cells from a related HLA matched donor. Results from this trial with seven patients included, and six that completed the treatment, were promising. Patients showed improvement in the phenotype and donor cells could be identified in skin and mucosa. It is, however, important to add that none of the patients was cured completely and while amelioration of the disease was achieved, results varied significantly between patients, with Patient 1 having only modest benefit and Patient 7 having recurrence in blistering after 60 days. Moreover, from seven included patients, one child died during the aggressive conditioning regimen, and one 183 days after the transplantation from subsequent infections showing mortality of this treatment to be above 25%. Mortality rates in bone marrow transplantation vary between indication, conditioning protocol and experience of treating center but for the mixed population with benign disorders and with reduced toxicity conditioning is estimated at around 15-20%.[105] Therefore the usage of systemic stem cell application should be further investigated and modified to achieve lower mortality rates. An approach with reduced toxicity conditioning together with the use of umbilical cord blood and additional infusion of mesenchymal stromal cells instead of bone marrow has been proposed.[87]

A place for revertant cell therapy

Despite the fact that many therapies for EB are in development, none of the proposed approaches has become a cure yet. For example, very promising gene therapy is impeded by safety issues and regulatory bodies while offering only treatment of a limited area of the body, while stem cell therapy in the form of hematopoietic stem cell transplantation using bone marrow does not completely cure the disease and has a high mortality rate. There is, therefore still a place for a safe and successful therapy for EB. In the next part of this chapter we will introduce the phenomenon of revertant mosaicism, a starting point for development of revertant cell therapy.

REVERTANT MOSAICISM – A NATURAL GENE THERAPY

In monogenetic disorders, mosaicism is a co-existence of two different cell populations within one organism, one containing a wild-type gene and one containing a mutated, disease causing, version of the same gene in one individual.[106] Mosaicism is usually an effect of a somatic mutation that occurs post-zygotically in one cell, which later gives rise to a larger cell population that can be identified.[107] Somatic mosaicism can be divided in forward and revertant mosaicism. We speak of forward mosaicism, if in a healthy organism a population of cells becomes affected by a genetic disease as a result of a somatic mutation that occurred post-zygotically. Revertant mosaicism (RM), however, describes the opposite situation, where a somatic mutation restores a wild-type phenotype in a population of cells in a diseased organism affected by a genetic disease. Thus forward somatic mosaicism induces a diseases phenotype in a cell population of a further healthy organism, while revertant mosaicism restores the healthy phenotype in an already affected organism (Figure 4) and therefore can be named natural gene therapy. Forward mosaicism as a cause of genetic disorders has been hypothesized for many years but proof of its existence at the DNA level only came in 1988 in the case of a boy with mild expression of ornithine transcarbamylase deficiency.[108] Many cases have been already described and forward mosaicism has been widely accepted as one of the origins of genetic diseases.[109] Recently, forward mosaicism was demonstrated in the mother of a child with DDEB.[110]

Revertant mosaicism is, regardless of increase in number of described cases, still thought to be a rare phenomenon. It has been observed for ages in plants, not as natural therapy of genetic disorders but as a variation of their color and symmetry. For example, the yellow color of maize is an effect of mutation inbreed, while the natural color is purple. One can quite often observe single purple segments or even spots within one segment, a result of reversion to the wild-type genotype by transposons.[111] The first case of revertant mosaicism as a mechanism of natural gene therapy leading to reversion to a wild-type phenotype in a human affected by genetic diseases was described in 1988, when the reverted HPRT gene was found in a patient with

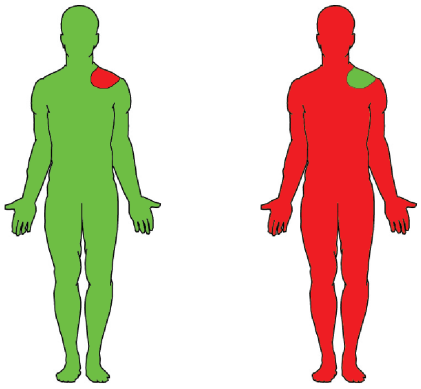
Lesch-Nyhan syndrome.[112] Since then, reversions have been found in other genetic diseases, and are observed in self-regenerating organ systems such as liver, blood and the skin.[113,114]
Reversion is a result of a genetic event and can occur due to different genetic mechanisms as gene conversion, intragenic (single and double) crossing over, back and second-site mutations. Second-site mutations can vary from single base pair substitutions, deletions or insertions to a large deletion of more than 2000 bp (Figure 5). As an effect of the second genetic correcting event, a wild-type protein, a protein with a different amino acid, or an aberrant, but partially functional protein, can be expressed. Surprisingly, more than one reversion mechanism can be present within a single individual. For example in one Wiskott-Aldrich Syndrom (WAS) patient due to a nonsense mutation in the WASp gene, 38 different reversion mechanisms were found[115]. The trigger for mutational DNA events leading to reversion is unknown.
The incidence of revertant mosaicism varies from around 11% in WAS,[116] 18% in Fanconi anemia,[117] 88% in tyrosinemia type I,[118] to presumably 100% in the subtype of JEB-nH caused by mutations in *COL17A1*.[119] Furthermore, additional cases are being constantly described, as recently in dystrophic EB (DEB),[120-122] ichthyosis with confetti[123] or X-linked lymphoproliferative disease.[124] This means that the realm of revertant mosaicism is expanding and its position changes from an exceptionally rare phenomenon towards a common aspect of genetic diseases.[125]

Revertant mosaicism in EB

The first case of a mosaic pattern in the skin was published in 1995 and described a woman, then 27 years old, affected by JEB-gen-intermed due to mutations in *COL17A1*, with patches of healthy looking skin distributed on her arms and hands.[56] The healthy looking skin could also withstand friction as opposed to its surrounding skin, where blisters could be easily provoked (Figure 4B). Immunofluorescence staining showed total absence of Col17 in the biopsy taken from the affected skin, while in the biopsy taken from one of the healthy looking patches, a linear staining for Col17 was seen in about 50 % of the basal cells (Figure 4B). Two years later, in 1997, Jonkman et al. found mitotic gene conversion to be the underlying mechanism of the phenomenon named revertant mosaicism.[126] The patient was heterozygous for a maternal frame shift mutation in exon 18 (c.1601delA) and paternal nonsense mutation in exon 51 (c.3676C>T). Most probably during mitosis of an epidermal stem cell a part of the paternal allele of *COL17A1* containing a healthy copy of exon 18 was moved to the maternal allele and covered the c.1601delA mutation. This resulted in one allele with a paternal mutation and one wild-type allele present in one of the cells present after the mitosis, which later gave rise to the patch of revertant skin.[126]

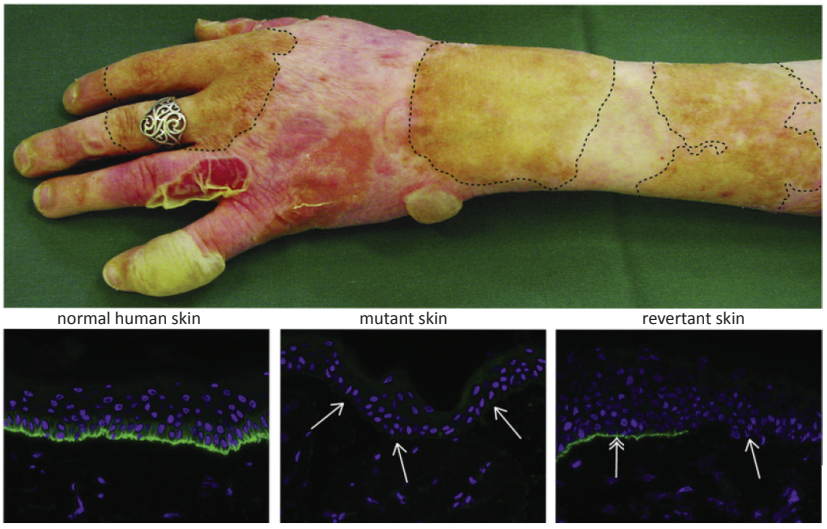
Figure 4 Graphical representation of somatic mosaicism and difference between forward and revertant mosaicism (A). On the left picture a skin patch with mutation (red) is present in an otherwise healthy body with a wild-type genotype (green). On the right picture situation is reversed, as an individual affected by genetic disease (red) has a healthy patch caused by revertant mosaicism (green). (B) shows a clinical picture of the patient with mutation in *COL17A1* presenting healthy looking, hyperpigmented skin patches (outlined) on her left arm surrounded by affected, blistering skin. Below pictures showing immunofluorescence staining for Col17, nuclei staining in blue and Col17 in green on all three pictures. On the left staining of a healthy control human skin showing normal expression of Col17; in the middle staining of the biopsy taken from this pateints affected skin with absence of Col17 (arrows) and to the right staining of the biopsy taken from the healthy looking skin with the partial expression of Col17 (double arrowhead). Part B of this figure is reproduced from Pasmooij et al. 2012 [129]; permission was obtained.

A



forward mosaicism revertant mosaicism

B



normal human skin mutant skin revertant skin

Current knowledge about revertant mosaicism in EB

Almost 20 years later, in 2014, from 18 genes causing EB, five already have been found to be reverted, totalling 34 described and published cases. An overview of the patients with revertant mosaicism per subtype of EB and affected gene is given in Table 1. With number of patients grew the number of different reversion mechanisms identified. Moreover, as in WAS, EB patients were found to have more than one revertant mechanism present in their body and each reversion event lead to a distinct revertant patch on the skin.[127] Furthermore, none of the four major types of revertant mechanisms found in EB seems to be favoured. In the revertant skin patches of EB patients, only keratinocytes were found to be revertant, whereas fibroblasts kept their mutant genotype.[128,129] There is still a lot to be discovered about RM, such as time of onset, possible growth of revertant skin and if some genes have a predisposition to become reverted.

In theory, a reversion in a stem cell during embryotic development would lead to a patchy skin pattern after birth with the same reversion mechanism present in each patch. Such a case, however, has not yet been described. If reversion occurs after diversion of an epidermal lineage, the earlier it happens, the larger the area of revertant skin should be, as it would grow exponentially with the skin. Interestingly, some patients reported that their skin healed later in life, one of them being a patient with *LAMB3* mutation, who indicated that his leg, earlier affected, became healthy during adulthood.[128] Beside the number of divisions of the reverted cell, one has to also consider the possible growth advantage or disadvantage. In the mouse model of EB simplex caused by a mutation in the gene encoding the epidermal intermediate filament, keratin 14 (KRT14) cells with the induced KRT14 mutation were overgrown by healthy keratinocytes suggesting that the healthy cells had an *in vivo* growth advantage.[130,131] It is therefore believed that for a revertant patch to become visible and recognisable, the following conditions have to be met:

- reversion must take place in an epidermal stem cell,
- reversion needs to occur early enough during epidermal development to have a chance to grow to a patch of visible size and/or growth of revertant cells *in vivo* is speeded by its biology.

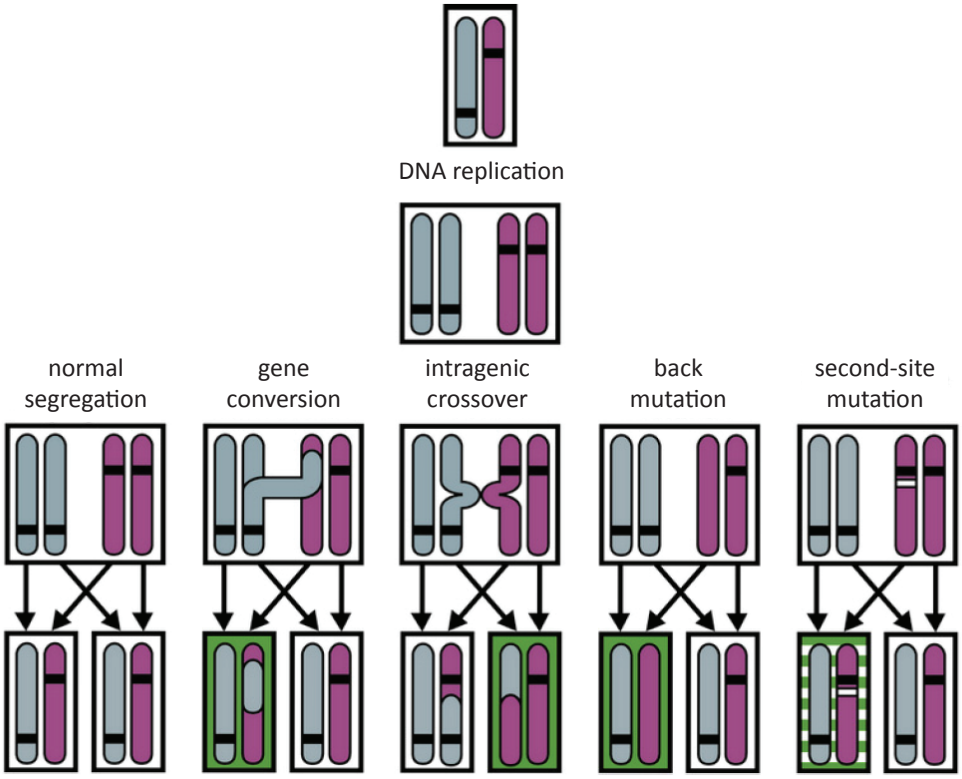


Figure 5 Different reversion mechanisms can correct an inherited mutation. In recessive disease, every cell contains two chromosomes, and the black bars indicate the positions of the mutations. With normal segregation each cell obtains one chromosome from the father and one from the mother. When DNA from one chromosome is non-reciprocally transferred to the other chromosome gene conversion occurs. In case this gene transfer is in the region containing the inherited mutation this mutation is lost in the daughter cells. One of the daughter cells will only carry one recessive mutation, and will therefore produce protein (green). Other reversion mechanisms, seen in hereditary skin diseases, are intragenic crossover, back mutation, and second-site mutation. Intragenic crossover results in one daughter cell with both inherited mutations on one chromosome, and another revertant daughter cell with one of the chromosomes without inherited mutations. In case of a second-site mutation a compensatory mutation is present in the same gene correcting the inherited mutation. The protein that is produced may be similar to the wild-type protein, or slightly aberrant although still functional (green/with stripes). Reused from Pasmooij et al with permission. Courtesy of dr AMG Pasmooij. [129]

It has also been suggested that no extraordinary mutation rate is needed for the reversion to occur and that the standard mutation rate is sufficient.[132] Studying old photographs of patients and comparing the revertant patches to the current situation have proved the stability of revertant patches in EB.[127] In a few cases, however, the growth of revertant patches have been claimed and Choate et al. showed that new revertant spots can arise during life in a patient with ichtyosis with confetti, disease caused by mutation in keratin 10 gene, KRT10.[123]

Clinical presentation of revertant mosaicism in EB

The presentation of revertant skin differs between types of EB, but lack of blistering is the common denominator. In patients with mutations in *COL17A1*, revertant skin contains hair and is darker than surrounding affected skin. This will be further elaborated upon in **Chapter 5** of this thesis. In patients with mutations in *LAMB3* or *COL7A1* lack of atrophy or erythema is an important sign but no difference in pigmentation can be seen. Revertant patches can also vary in size, from 2 to about 10 centimetres in diameter. The number of revertant patches per patient may also differ, from one to multiple covering up to around 10% of the total body surface. As for now, the youngest patient was 10 years old when his revertant patch was identified.

The concept of revertant cell therapy

Revertant cell therapy is a logical step forward. The cultured epidermal grafts composed of revertant keratinocytes might be used to cover wounds. The technique for cultured skin grafting is available and used for treatment of burns and leg ulcers.[8,27] Keratinocytes, and their stem cells, are affected by mutations causing EB and thus can also become naturally corrected in patients with revertant mosaicism. Therefore in this thesis we propose an approach to treat cutaneous manifestations of EB by autologous transplantation of revertant keratinocytes and their stem cells. This approach does not require genetic manipulation, introduction of recombinant proteins or usage of allogeneic material.

AIMS AND OUTLINE OF THIS THESIS

The general aim of this thesis was to translate the phenomenon of RM in to revertant cell therapy, which could benefit the population of EB patients. In **Chapter 2** we describe a human pilot study of transplantation of revertant epidermal grafts in a patient with JEB-gen-intermed due to mutations in *COL17A1* gene. **Chapter 2** shows an elegant technique to prepare a wound bed of the acceptor site by removing the affected mutant skin using the natural pathological mechanism of the disease that causes splitting of the epidermis in the lamina lucida. The transplantation was surgically successful, but skin fragility did not improve since the graft contained insufficient revertant cells. Decrease in the percentage of revertant cells is the main focus of **Chapter 3**, which describes a murine model of revertant cell therapy. We follow the revertant keratinocytes from the moment that the skin biopsy is taken, through the graft production up to 16 weeks after transplantation on mice. **Chapter 3** gives thus more insight in the reason why the procedure described in **Chapter 2** was not successful and suggests the underlying mechanism. Based on experience from chapters 2 and 3 we then took a step back and used grafting of punch biopsies instead of cultured epidermal grafts in a patient with mutations in *LAMB3* gene. This procedure, described in **Chapter 4**, was successful and led to expansion of the revertant area of the patient's body. Although during the experiments described in **Chapters 2-4** we tried to move from the bench to the bedside, the encountered differences in pigmentation of revertant patches between subtypes of EB made us wonder if proteins involved in the dermal epidermal junction also influence pigmentation. Therefore in **Chapter 5** the translation process was reverted and we moved from the bedside to the bench and looked into the pigmentation and melanocyte distribution in the revertant and mutant skin of revertant patients with mutations in *COL17A1*, *LAMB3* and *COL7A1*, which resulted in interesting findings adding to the understanding of melanocyte biology. **Chapter 6** describes three experiments that did not lead to a publication but could be a basis for future experiments and development of in the development of the revertant cell therapy. Briefly, we used of flow cytometry for selection of Col17 revertant keratinocytes, attempted to transplant Col7 revertant human keratinocytes in a murine model, and performed a clinical pilot to transplant revertant keratinocytes in suspension. Finally, in **Chapter 7** our findings are discussed and confronted with the newest developments in the therapy for EB and regenerative medicine. In this chapter we also propose future studies needed to develop revertant cell therapy, not only for EB but also for other inherited genetic diseases.

TABLE 1

Patients with epidermolysis bullosa and revertant mosaicism. Legend: aa, amino acids; Col7, type VII collagen; Col17, type XVII collagen; KRT14, keratin 14; Lam-332, laminin-332; N/D, not determined. Based on table by Pasmooij et al.,[129] modified and updated.

Subtype of EB	Gene	Number of patients	Mutations	Reversion mechanism	Effect on protein	Reference
Recessive EB Simplex	KRT14	1	c.526-2A>C; c.526-2A>C	N/D	KRT14 with deletion of two aa and one different aa (p.Ile176Met)	133
EB Simplex generalized severe		1	p.Arg-125Cys	c.242insG	Ablation of KRT14 with the c.Arg125Cys mutation	134
JEB-gen-in-termed	COL17A1	12	c.2237delG; c.2237delG	c.2263+2T>C	Col17 with deletion of 12 aa	56,126, 135-137
			c.1601delA; p.Arg1226X	gene conversion c.3677G>C	wt Col17 Col17 with one different aa (p.Arg1226Ser)	
			c.2237delG; p.Arg1226X	c.2228-101_2263+70del ins15 c.2259_2263+9del c.2263+2T>C	Col17 with deletion of 12 aa Col17 with deletion of 12 aa Col17 with deletion of 12 aa	
			c.2237delG; p.Arg1226X	c.2263+2T>C	Col17 with deletion of 12 aa	
			p.Arg1226X; c.4320insC	3676T>C or gene conversion c.4358-1G>A	wt Col17 Col17 of correct size with a stretch of 13 different aa	

Subtype of EB	Gene	Number of patients	Mutations	Reversion mechanism	Effect on protein	Reference
			c.2237delG; c.2237delG	c.2227+153_2336-318del c.2238C>T	Col17 with deletion of 36 aa Col17 with deletion of 12 aa	
			c.2237delG; c.2237delG	N/D	N/D	
			c.1179delA; c.3327delT	N/D	N/D	
			c.3131delC; c.3131delC	N/D	N/D	
			c.1260delC; c.3495-3496delCT	N/D	N/D	
			c.3898-3899delTC; c.3898-3899delTC	c.3973-3974dupGG	Col17 with insertion of 25 aa	138
			N/D	N/D	N/D	139
	LAMB3	2	c.628G>A (p.Glu210 Lys); p.R635X	c.596G>C (p.Gly199Ala) c.628+42G>A	Lam-332 with two different aa (p.Gly199Ala and p. Glu210Lys) Lam-332 with insertion of 22 aa	128

Subtype of EB	Gene	Number of patients	Mutations	Reversion mechanism	Effect on protein	Reference
			c.628G>A (p.Glu210 Lys); c.628G>A	c.619A>C (p.Lys207Gln) c.629-1G>A c.565-3T>C	Lam-332 with two different aa (p.Lys207Gln and p.Glu210Lys) Lam-332 with deletion of 22 aa Lam-332 with one different aa (p.Glu210Lys)	
Recessive DEB	COL7A1	9	p.Arg578X; c.7786delG	intragenic crossingover	wt Col7	120
			c.6527dupC; c.6527dupC	single nt deletion c.6528delT	wt Col7	140
			p.Gln2170X; p.Gln2170X	c.6510G>T	Col7 with one different aa (p.Gln2170Tyr)	122
			c.425A>5; c.8206G>A	Mitotic recombination: c.425A>G absent	Wt Col7	141
			c.2142A>G; c.6527dupC	Second-site mutation: c2144A>G	substitution p.Tyr-715Cys	
			c.884delG; c.6527dupC	Back mutation/mitotic recombination: c.884delG absent	Wt Col7	
			c.425A4G; c.425A4G	Second-site mutation: c.426+3G>A	Wt Col7	

Subtype of EB	Gene	Number of patients	Mutations	Reversion mechanism	Effect on protein	Reference
			c.425A4G; c.1837C>T	Mitotic recombination is suspected with both mutations present in one allele and other being a wild-type (no molecular proof)	Wt Col7	
			c.2894C>T; c.6176A>G	Back mutation/mitotic recombination: c.6176A>G absent	Wt Col7	
Dominant DEB	COL7A1	1	c.6127G>A; N/A	Back mutation/mitotic recombination: c.6127C>A absent	Wt Col7	141
Kindler syndrome	FERMT1	8	N/D	N/D	N/D	142
			c.676dupC; c.676dupC	transcriptional slippage or RNA editing	Wt kindling 1	143
			c.456dupA; c.456dupA	slipped mispairing and mitotic recombination	Wt kindling 1	144
			c.676dupC; c.676dupC	slipped mispairing and mitotic recombination	Wt kindling 1	
			c.676dupC; c.676dupC	N/D	N/D	
			c.676dupC; c.676dupC	N/D	N/D	
			c.676dupC; c.676dupC	N/D	N/D	
			c.676dupC; Trp559X	N/D	N/D	

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