Stairways to Advanced Therapies for Epidermolysis Bullosa

Laura De Rosa,1,3 Elena Enzo,2,3 Michele Palamenghi,2,3 Laura Sercia,2,3 and Michele De Luca2

1Holostem Terapie Avanzate, S.r.l., 41125 Modena, Italy
2Centre for Regenerative Medicine “Stefano Ferrari,” University of Modena and Reggio Emilia, 41125 Modena, Italy
Correspondence: michele.deluca@unimore.it

Epidermolysis bullosa (EB) is a devastating genetic skin disease typified by a plethora of different phenotypes and ranking from severe, early lethal, to mild localized forms. Although there is no cure for EB, recent progress in pharmacology and molecular and cellular biology is boosting the development of new advanced therapeutic strategies. Here we will focus on two main categories of such therapies: (1) those aimed at controlling inflammation and inducing reepithelialization of the wounds, and (2) those, perhaps more challenging and ambitious, that aim to permanently regenerate a fully functional epidermis, which requires targeting of epidermal stem cells. In both cases, the genetic variants underlying the different EB forms and factors, such as genetic background, modifier genes, comorbidities, and lifestyle, all of which impinge on EB genotype-phenotype correlation, need to be defined.

Inherited epidermolysis bullosa (EB) is characterized by recurrent blistering of stratified epithelia. EB is caused by more than 1000 mutations in at least 16 structural genes encoding proteins forming hemidesmosomes and anchoring fibrils, which are essential for the integrity of the epidermal–dermal junction (Has et al. 2020a). Blisters arise spontaneously or upon minimal trauma as the result of the fragility of skin and mucous membranes. Common features of many EB forms include damage of ocular surface, upper airways, oral mucosa and gastrointestinal and renal systems, as well as hair, nail, and enamel defects (Bardhan et al. 2020; Has et al. 2020a). Incidence and prevalence of EB in the United States are 11.1 per one million people and 19.6 per one million live births, respectively. Similar values have been reported in Europe (Fine 2010; Has et al. 2020a). EB affects individuals from all ethnic origins regardless of gender and displays either dominant or recessive patterns of inheritance. EB phenotypes range from mild, localized blistering to massive blistering, erosions and chronic wounds. Severe EB forms can be early lethal and generalized EB frequently leads to aggressive squamous cell carcinoma (SCC) (Condorelli et al. 2019; Bardhan et al. 2020).

**EB FEATURES**

Based on the plane of ultrastructural fragility, EB is classified into four major types: (1) EB simplex
(EBS), defined by intraepidermal blistering; (2) junctional EB (JEB), characterized by blistering within the lamina lucida of the basement membrane; (3) Kindler EB (KEB), defined by variable levels of tissue cleavage; and (4) dystrophic EB (DEB), where blisters occur in the dermis, just below the lamina densa of the basement membrane (Fig. 1A; Bardhan et al. 2020). The severity of skin clinical manifestation correlates to extent of blistering—localized to generalized—wound depth, and scarring, ranging from mild to severe (Fig. 1B). Being superficial, EBS blisters usually heal without significant scarring (Pfendner and Lucky 1993). In contrast, inter-
mediate to severe, generalized JEB and DEB are marked by persistent inflammation, fibrosis, and scarring. In particular, the depth of DEB lesions is associated to altered wound healing processes and severe scarring that usually lead to highly disabling syndactyly (see below) (Fine et al. 2014; Condorelli et al. 2019).

EB Simplex

EBS is the most common form of EB (~1 case per 25,000 live births). Although it can arise from mutations in seven different genes (Has et al. 2020a), over 75% of EBS is due to dominantly inherited mutations affecting KRT5 and KRT14, the genes encoding keratin 5 (K5) and keratin 14 (K14), which form the intermediate filament network of basal keratinocytes (Bardhan et al. 2020; Has et al. 2020a). Their alteration leads to keratinocyte cytolysis (Coulombe and Lee 2012). Mutations in highly conserved amino acids within the helix initiation or termination motifs lead to severe EBS, which is characterized by blisters, often leading to chronic erosions, covering the entire skin surface and affecting several mucous membranes (Coulombe et al. 2009). Aminoacidic substitutions in other K5/K14 regions lead to localized EBS, marked by milder clinical manifestations usually restricted to the extremities (Coulombe and Lee 2012). Very rare, severe (in some cases lethal) forms of EBS are caused by recessively inherited nonsense or missense KRT5 and KRT14 pathogenic variants (Fine et al. 1989; Has et al. 2020a). EBS can also be caused by mutations in plectin (encoded by PLEC) and dystonin (encoded by DST), which are hemidesmosomal proteins that anchor keratin filaments to the plasma membrane (Fig. 1A; Bardhan et al. 2020; Has et al. 2020a).

Junctional EB

JEB is one of the most devastating forms of EB. It is due to recessively inherited mutations in genes encoding the heterotrimeric protein laminin 332 (LAMA3, LAMB3, LAMC2), collagen XVII (COL17A1), integrins α6β4 (ITGA6, ITGB4), and integrin α3 (ITGA3) (Fig. 1A). The most severe forms of JEB are caused by mutations in LAMA3, LAMB3, LAMC2, ITGA6, and ITGB4, while mutations in COL17A1 (and ITGA3) have a milder phenotype (Bardhan et al. 2020). Patients carrying biallelic premature termination codons leading to absence of laminin 332 or α6β4 (severe JEB) usually die within 2 years after birth (Pfendner and Lucky 1993; Has et al. 2020a). Approximately 40% of patients with intermediate JEB die before adolescence, while adults have a high risk of developing SCC (Malipieddi et al. 2004; Fine et al. 2008; Yuen and Jonkman 2011). Missense or splicing mutations that allow residual expression of the protein, even if truncated and only partially functional, can significantly reduce the severity of the phenotype (Condrat et al. 2019), suggesting that low expression of one component can still sustain its interactions with the binding partners (Kiritsi et al. 2011).

Kindler EB

KEB is caused by mutations in FERMT1, the gene encoding fermitin family homolog 1 (kindlin-1), an intracellular protein of focal adhesions (Fig. 1A; Bardhan et al. 2020; Has et al. 2020a). Blisters occur at different levels of the epidermal–dermal junction: within the basal keratinocyte, along the lamina lucida, and below the lamina densa of the basement membrane (Fig. 1A; Bardhan et al. 2020). Features include skin fragility and mild photosensitivity, both improving with age, poikiloderma, palmoplantar hyperkeratosis, and high risk of developing SCC in adulthood (Emanuel et al. 2006). As with other EB forms, several mucous membranes, such as urethral, anal, esophageal, and genital mucosae, are involved (Bardhan et al. 2020).

Dystrophic EB

DEB can be dominantly or recessively inherited (Bardhan et al. 2020) and it is due to over 200 mutations in COL7A1 (Varki et al. 2007), the gene encoding collagen VII (C7), the main component of anchoring fibrils (Fig. 1A; Sakai et al. 1986).

Dominant DEB (DDEB) has a mild phenotype with blisters primarily involving the ex-
tremities, which often results in scarring and nail loss (Das and Sahoo 2004). DDEB is frequently caused by heterozygous glycine substitutions within the collagenous triple helix domain (Christian et al. 1996). Monoallelic recurrent splice-site or indel COL7A1 mutations lead to in-frame skipping of entire exons (e.g., exon 87) (Jiang et al. 2002; Schwieger-Briel et al. 2015), or large deletions within the triple-helical domain (Chmel et al. 2018). Self-improving DDEB has been associated with skipping of specific exons (e.g., exon 36) (Christian et al. 1997) or specific glycine substitutions (Fassihi et al. 2005; Shi et al. 2015).

In contrast, recessive DEB (RDEB) can be devastating, being characterized by severe blistering, massive scarring, and disabling joint contractures and pseudo-syndactyly (Varki et al. 2007), all of which highly reduce the patients’ quality of life (Pfendner and Lucky 1993; Tang et al. 2021). Adults with generalized RDEB almost invariably develop aggressive, highly metastatic SCC (Fine et al. 2009). Severe RDEB usually results from biallelic COL7A1 premature termination codons (Uitto and Christiano 1994), but variants include nonsense or splice site mutations, deletions, or insertions, “silent” glycine substitutions or non-glycine missense mutations within triple helix or non-collagenous NC-2 domains. The nature and the positions of these mutations correlate with the severity of the phenotype (Varki et al. 2007). Specific glycine and arginine substitutions have been implicated in RDEB inversa (van den Akker et al. 2011).

**Additional Factors Defining EB Genotype–Phenotype Correlation**

EB genotype–phenotype correlation is an intriguing mash up of different factors, such as specific mutations, modifier genes, genetic background, lifestyle, and comorbidities. For instance, digenic variants of KRT5 and KRT14 (Kim et al. 2017), or double homozygous mutations of EXPH5 (encoding exophilin 5) and COL17A1 (Vahidnezhad et al. 2018) or PLEC1 and ITGB4 (Kariminejad et al. 2019) have been associated with unexpected phenotypes. Peculiar phenotypes can also be explained by modifier genes and/or their genomic variants, whose products may modulate or influence EB proteins (Nyström et al. 2021). For instance, variants of the promoter of MMP1, which encodes a metalloproteinase that digests C7, are associated with a higher RDEB severity because of the imbalance between C7 synthesis and degradation (Titeux et al. 2008). Incomplete EB penetrance can be ascribed to postzygotic mosaicism for a disease-causing variant (Shipman et al. 2014; van den Akker et al. 2015; Vázquez-Osorio et al. 2017) or to spontaneous revertant mosaicism (RM) (Jonkman et al. 1997; Darling et al. 1999; Smith et al. 2004; Pasmooj et al. 2007, 2010, 2012; Kiritsi et al. 2012; Lai-Cheong et al. 2012). Different genetic backgrounds (also among relatives), comorbidities, or other genetic disorders lead to complex, apparently “new” phenotypes (Maccari et al. 2019). Individual (e.g., personality, family context), socioeconomic (e.g., access to medical care and hygienic conditions), and environmental conditions (e.g., climate) might also affect EB penetrance.

A complete EB database taking into account all of the genetic, molecular, biological, and clinical parameters described above would be a great benefit to assist researchers and physicians in setting up patients’ stratification and personalized therapeutical strategies (Uitto et al. 2021).

**Wound Healing in EB**

Altered wound healing is a key player in the pathogenesis of EB. Unbalance of the sequential and partly overlapping four wound healing phases—hemostasis, inflammation, cell proliferation, and tissue remodeling—may result in two opposite detrimental outcomes: a delay or lack of wound closure and the formation of a hypertrophic scar (Gurtner et al. 2008). Multiple factors, insisting mainly on the last three phases of wound healing, variably undermine wound healing and repair processes and underpin both chronic wounds and fibrosis observed especially in severe EB forms (Pfendner and Lucky 1993; Fine et al. 2014; Cianfarani et al. 2017; Tartaglia et al. 2021). Skin fragility and recurrent blisters cause persistent inflammation, delayed reepithelization, and nonhealing ulcers, which become easily infected, further protracting the healing pro-
Toward Tailored EB Therapies

The prospective of a definitive therapy tackling clinical manifestations of EB is still elusive. Most of the current therapeutic approaches are palliative and focused on wound care and control of symptoms (Goldschneider et al. 2014; Bruckner-Tuderman 2019; Prodinger et al. 2019). Because of the heterogeneity of EB, likely there would not be a single successful therapeutic strategy and single or combined treatments should be tailored to different categories of EB patients.

Hereafter we will focus on emerging innovative treatments, aimed at either relieving symptoms and inducing a more stable reepithelialization, or targeting the genetic defects and/or replacing the epidermis by transgenic stem cells, all of which did not yet attain the level of a standardized therapy available to all EB patients.

RELIEF OF SYMPTOMS AND/OR INDUCTION OF REEPITHELIALIZATION

Drugs

Different molecules have been proposed to reduce inflammation and fibrosis and foster wound healing. Oleogel-S10 (NCT01294241, NCT03068780), consisting of birch bark–derived triterpenes, can improve wound healing through modulation of inflammatory mediators and stimulation of keratinocyte migration and differentiation (Fig. 2A1; Kern et al. 2019; Schwieger-Briel et al. 2019). Apremilast, a phosphodiesterase-4 inhibitor, and diacerin (NCT03154333), an anthraquinone derivative inhibiting interleukin-1β, inhibit proinflammatory and stress-response pathways, and are used to reduce blistering in EBS patients (Fig. 2A1; Prodinger et al. 2019). Animal studies demonstrated that losartan, an angiotensin II antagonist originally approved for the treatment of hypertension, inhibits TGF-β and reduces inflammation and fibrosis (Nystrom et al. 2015, 2021; Inamadar 2020). A phase I/II clinical trial on RDEB patients (EudraCT: 2015-003670-32) have confirmed losartan’s safety and suggested its clinical efficacy (Kiritsi 2020). A phase III clin-
ical trial is needed to confirm losartan’s safety and efficacy, also in pediatric patients, hence to allow its routine use at least in severe RDEB (Fig. 2A1).

Recently, drug-mediated modification of genetic variants has been endorsed. The aminoglycoside gentamicin can induce translational read-through of premature termination codons in \textit{COL7A1}, \textit{COL17A1}, and \textit{LAMB3}, both in vitro and in vivo, when administered topically or intravenously (Cogan et al. 2014; Woodley et al. 2017; Lincoln et al. 2018; Hammersen et al. 2019; Kwong et al. 2020; Li et al. 2020; Hung et al. 2021). Gentamicin aims at replacing the premature termination codon with a novel amino acid, hence restoring, at least temporarily, a functional protein. Ongoing safety studies will determine optimal dosage, treatment intervals and cumulative toxicity profile (NCT04140786, NCT03392909, NCT04644627, NCT02698735, NCT03012191, NCT03526159) (Fig. 2A2).

**Protein and Cell Therapies**

Mouse models have suggested that recombinant C7, administered either topically or systemically, homes to the epidermal–dermal zone, promotes RDEB wound healing, and prevents blister formation, both in skin and mucous

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**Figure 2.** Current advanced therapies for epidermolysis bullosa. (A) Protein replacement and reepithelialization (1–6), and (B) epidermal replacement (7–9). Therapies are displayed according to our view on the level of complexity of the therapeutic approach.
membranes (Woodley et al. 2004, 2013; Remington et al. 2009). A phase I/II clinical trial of systemically administered recombinant C7 in RDEB patients was implemented (NCT03752905), but no evidence of clinical efficacy has yet been reported (Fig. 2A3).

Cultures of allogeneic or transgenic autologous fibroblasts have been injected intradermally in RDEB patients (Wong et al. 2008; Georgiadis et al. 2016; Lwin et al. 2019) (NCT01749306, NCT02493816, NCT02810951, NCT04213261). However, the injection of fibroblasts in skin wounds caused diffuse erythema and in some cases pain (NCT02810951, NCT02493816). The limited life span of fibroblasts is likely to require multiple periodical injections, which, in our opinion, could hamper their use as a routine treatment of severe generalized RDEB and might limit their application to small, defined RDEB areas (Fig. 2A4).

Delivery of RNA or DNA Molecules and In Vivo Gene Therapy

New therapies based on antisense oligonucleotides (AONs), short interfering RNA (siRNA), or spliceosome-mediated mRNA trans-splicing (SMaRT) have been recently investigated. AONs aim to restore an open reading frame through the skipping of the mutated nonessential exon (Turczyński et al. 2012; Bremer et al. 2016; Kowalewski et al. 2016; Bremer et al. 2019). An AON designed to target exon 73 of COL7A1 is formulated for topical application and is currently in a phase I/II clinical trial (NCT03605069) (Fig. 2A5; Bornert et al. 2021). SMaRT aims at replacing mutated sequences of a pre-mRNA transcript with wild-type sequences supplied by an exogenous engineered RNA molecule (Fig. 2A5; Bauer et al. 2013; Peking et al. 2016; Tockner et al. 2016; Liemberger et al. 2018; Peking et al. 2019), while siRNAs specifically aim at tackling dominant forms of EBS and DEB (Fig. 2A5; Atkinson et al. 2011; Pendaries et al. 2012). Although these oligonucleotides can be easily delivered to skin cells in open wounds, their use at the time in non-wounded RDEB skin might be challenging and require cationic liposomes (Desmet et al. 2016a,b). In this light, a highly branched poly(β-ester) polymer (HPAE) for delivering COL7A1 cDNA has been recently developed for intradermal injection or topical application and has shown restoration of C7 expression in mouse models (Fig. 2A6; Zhou et al. 2016; Zeng et al. 2021).

A rather promising in vivo gene therapy strategy envisages the topical delivery of COL7A1 cDNA using a modified replication-deficient human herpes simplex virus type 1 vector (HSV-1) formulated into a gel (med.stanford.edu/dermatology/research/research/currentstudies/krystal.html) (Fig. 2A6). This strategy is quite appealing, since it would not require expensive, cumbersome, and time-consuming manufacturing processes or invasive clinical procedures. A phase I/II clinical trial clearly demonstrated both synthesis and deposit of C7 and stimulation of wound closure in pediatric and adult RDEB patients (NCT03536143) (Gurevich et al. 2022). Noteworthy, the episomal non-integrating nature of HSV-1 makes repeated—perhaps lifelong—application necessary (Has et al. 2020b; Welponer et al. 2021). In this light, data on long-term clinical efficacy and safety of repeated HSV-1 administration are not yet available. In addition, no evidence of efficacy on large wounded areas was reported, leaving an open question on its potential use on severe RDEB. Ongoing phase III clinical trials (NCT04491604, NCT04917874) would hopefully answer some of these questions.

EPIDERMAL REPLACEMENT

All the therapeutical strategies described above, some of which are quite promising and in phase I–III clinical trials, have a common drawback, namely, they could likely have only a temporary beneficial effect. The notion that (1) the many proteins forming the epidermal–dermal junction are solely synthesized by basal keratinocytes (with the notable exception of C7, which is in part produced by dermal fibroblasts) (Ryynänen et al. 1992), (2) the epidermis is a highly renewing tissue, and (3) proteins have a limited, defined half-life, implies that a definitive restoration of the epidermal–dermal junction requires the permanent correction of the epidermis. This goal can be achieved only through epidermal stem cells. Would genetic correction of fibroblasts be suffi-
GENETIC MODIFICATIONS FOR EB TREATMENT

Spontaneous Revertant Mosaicism

RM refers to a stochastic event in which corrective mutations occur on the mutated allele and leads to spontaneous gene therapy. Such a phenomenon has been discovered when a blistering body site of a COL17A1-JEB patient inexplicably showed limited areas of nonblistering, apparently normal skin (Jonkman et al. 1997). Since then, RM has been reported virtually in all EB forms (Fig. 2B7; Pasmooij et al. 2012), but the frequency of its occurrence is unclear. As most recessively inherited EB carries compound heterozygous mutations, confirmation of RM is necessary before expansion. Identification of RM is challenging, but recently a method has been proposed based on Cas9 enrichment and long-run nanopore sequencing that identify revertant correction resulting from homologous recombination (Natsuga et al. 2022). The genetic mechanism behind this phenomenon remains uncertain but it has been associated with back mutations, mitotic recombination, and novel mutations during cell division (Kiritsi et al. 2014). RM occurs mostly in keratinocytes, which, evidently, proliferate and generate patches of phenotypically normal skin. Thus, it has been suggested that revertant clonogenic keratinocytes could be used to prepare autologous cultures to be grafted onto the wounds of the patient (Pasmooij et al. 2012). A proof-of-principle on the potential use of such cultures has been obtained (Gostyński et al. 2014; Matsumura et al. 2019) and a clinical trial for RDEB has been implemented in Japan (UMIN:000020734) (Fig. 2B7).

Combined Ex Vivo Cell and Gene Therapy

Cultures of human clonogenic keratinocytes generate epithelial grafts that have been extensively used in many clinical settings (De Luca et al. 2006, 2019). Thus, genetic modification of epidermal keratinocytes can be used to prepare transgenic grafts able to restore a functional epidermis in EB patients (De Rosa et al. 2020). Genetic modification of keratinocytes can envisage either the use of integrating vectors carrying a corrected copy of the gene (Fig. 2B8) or the editing of the mutated gene (Fig. 2B9). The first strategy can suit recessively inherited forms of EB, while gene editing is required to correct either dominant and recessive mutations. The CRISPR/Cas9 technology is under investigation in keratinocytes, fibroblasts, or induced pluripotent stem cells (iPSCs) for gene editing of many forms of EB (Fig. 2B9; Kocher and Koller 2021; Kocher et al. 2021; O’Keeffe Ahern et al. 2021; Ramovs et al. 2021). Base editing is emerging as potentially suitable for correcting EB point mutations (Osborn et al. 2018, 2020; Rees and Liu 2018). The refinement of base editing in the form of “prime editing” represents a further progress, potentially able to edit the vast majority of all pathogenic EB mutations (Fig. 2B9; Anzalone et al. 2020).

Gene editing approaches are largely at the preclinical stage. Once fully developed, they could also be applied to recessively inherited EB, provided that their efficiency in targeting epidermal stem cells (see below) would be comparable to that of gene addition strategies.

Hereafter, we will focus on gene therapy strategies that are already in clinical trials.

Generalized intermediate LAMB3-JEB was the first genodermatosis successfully tackled by combined ex vivo cell and gene therapy. The first patient received, on the anterior part of his upper legs, ~500 cm² of autologous cultured keratinocytes transduced with a γ-retroviral vector (γRV) carrying a LAMB3 cDNA (Mavilio et al. 2006). Afterward, two LAMB3-JEB patients were successfully grafted using γRV-LAMB3-transduced keratinocytes (Bauer et al. 2017; Hirsch et al. 2017). Despite genetic and clinical differences, all three patients presented a stable, fully functional epidermis after a very long-term follow-up (6–16 years) (De Rosa et al. 2020; Kueckelhaus et al. 2021). No adverse events have been reported in those three patients, who received ~4 × 10⁸ trans-
genic clonogenic keratinocytes (De Rosa et al. 2020).

Noteworthy, transgenic epidermal grafts have proven to be lifesaving in the last LAMB3-JEB patient, a 7-year-old boy affected by a severe form of the disease (Hirsch et al. 2017). The child suffered from complete epidermal loss on ~80% of his total body surface and had a very poor prognosis. Virtually his entire skin was fully restored by ~1 m² of transgenic cultured epidermis (Hirsch et al. 2017). During the entire 6-year follow-up, his transgenic epidermis did not (and still does not) develop blisters. The transgenic skin is resistant to mechanical stress, heals wounds normally, does not itch, has normal mechanoreception and nociception, does not need ointments, and expresses normal levels of laminin 332 properly located at the epidermal–dermal junction. Thus, the child’s quality of life is greatly improved (Kueckelhaus et al. 2021).

An oncoming multicenter European phase II/III clinical trial (referred to as Hologene 5) aims to confirm safety and efficacy of transgenic epidermal cultures on a larger number of LAMB3-JEB patients (NCT05111600) (Fig. 2B8; De Rosa et al. 2021).

Autologous cultured keratinocytes transduced with a γRV carrying a COL7A1 cDNA (earlier referred to as LEAES and today as EB-101), have been used to restore the expression of C7 on 42 skin wounds on seven RDEB patients (NCT01263379) (Siprashvili et al. 2016). At 2-year follow-up, more than 70% of the treated wounds healed and expressed C7 assembled in functional anchoring fibrils on at least 50% of their surface, all of which significantly improved the clinical picture (Fig. 2B8; Eichstadt et al. 2019). No adverse events related to the use of γRV-corrected cells were reported (Eichstadt et al. 2019). An ongoing phase III clinical trial aim to confirm safety and efficacy on a larger number of RDEB patients (NCT04227106). We obtained similar results in a similar phase I/II trial (unpubl. data) (NCT02984085). Other evidence should emerge from an ongoing clinical trial envisaging the use of transgenic skin equivalents, in which both RDEB keratinocytes and fibroblasts are transduced with a SIN-γRV carrying COL7A1 (EBGraft) (NCT04186650) (Fig. 2B8).

While the LAMB3-transgenic epidermis exhibits a fully functional, seamless basement membrane and a normal number of mature hemidesmosomes (Mavilio et al. 2006; De Rosa et al. 2014; Bauer et al. 2017; Hirsch et al. 2017; Kueckelhaus et al. 2021), COL7A1-transduced keratinocytes were able to partially restore the expression of C7, hence they regenerated a sort of “mosaic” patterned epidermis (Siprashvili et al. 2016; Eichstadt et al. 2019; our unpubl. data). This difference could be, at least in part, ascribed to a lower transduction efficiency of γRV-COL7A1 (Siprashvili et al. 2016), as compared to γRV-LAMB3 (Mavilio et al. 2006; De Rosa et al. 2014; Bauer et al. 2017; Hirsch et al. 2017), and to competition between untransduced and transgenic RDEB keratinocytes, which is unlikely to occur in the JEB scenario. In fact, signals emanating from the interaction of laminin 332 with integrins α6β4B3 induce nuclear localization of the Yes-associated protein (YAP), a transcriptional coactivator instrumental in sustaining human epidermal stem cells (Schlegelmilch et al. 2011; Zhang et al. 2011; Walko et al. 2017; De Rosa et al. 2019). LAMB3-JEB triggers YAP inactivation and leads to epidermal stem cell depletion, supporting the notion that JEB is an adhesion and a stem cell disease (De Rosa et al. 2019). It follows that genetic correction of LAMB3-JEB rescues not only cell adhesion but also epidermal stemness, thus conferring to transgenic JEB keratinocytes a selective advantage over the untransduced counterpart, both in vitro and in vivo (De Rosa et al. 2019). Such a selective advantage does not hold true for RDEB clonogenic keratinocytes. This hurdle might be exceed- ed by a substantial improvement of the efficiency of RDEB keratinocyte transduction.

Regardless of the strategy used to genetically correct EB keratinocytes, combined ex vivo cell and gene therapy would meet the remarkable goal of achieving a definitive, permanent restoration of the skin, but it has many drawbacks: specific skills are required for proper cultivation and transduction of epidermal cells, which need an approved facility for good manufacturing practices (GMPs) and compliance with regulations for advanced therapy medicinal products (ATMPs) (Pellegrini et al. 2013, 2016, 2018; Magrelli et al. 2020). Thus, the preparation of trans-
genic grafts is expensive and needs highly qualified centers and the involvement of the industry. Furthermore, the transplantation of the cultures requires surgical procedures, which, however, would not be repetitive for the treated areas. Finally, although intense research is ongoing on the potential use of epithelial cultures for the regeneration of internal mucous membranes—a goal already attained with the ocular surface (Pellegrini et al. 1997, 2013; Rama et al. 2010) and, partially, with the urethral epithelium (Romagnoli et al. 1990, 1993; Corradini et al. 2016; Sceberras et al. 2020; Maurizi et al. 2021)—to date this technology can tackle only skin lesions.

Even though progressive replacement of diseased epidermis in adult patients is feasible, in our opinion children would be the ideal target of transgenic grafts: children’s cultures have a higher clonogenic potential, a lower number of grafts is needed to cover their body surface, and it would be easier to prepare the receiving bed. In fact, a bank of transgenic epidermal stem cells taken from these patients at birth can be created, which can be used to treat, step-by-step, skin lesions when they develop, thus preventing, rather than restoring, the severe skin clinical picture developing through adulthood.

EPIDERMAL STEM CELLS, THE CORNERSTONE FOR EPIDERMAL REPLACEMENT

Given the complexity and the cost of the technology (and to a certain extent of the surgical procedure), in our opinion epidermal replacement can be justifiable if it underpins definitive and permanent restoration of a functional epidermis after a single transplantation on a given body site. This notion raises a crucial issue, namely, the correction of epidermal stem cells as a mandatory cornerstone for an appropriate epidermal replacement. In fact, the remarkable clinical outcomes obtained both in cell and gene therapy, with either hematopoietic or epithelial tissues, have a common denominator, which is the corresponding stem cell as the “active substance” of those ATMPs (Aiuti et al. 2009; Rama et al. 2010; Biasco et al. 2016; Hirsch et al. 2017; De Luca et al. 2019).

Holoclone-Forming Keratinocytes: Drivers of Human Epidermal Maintenance and Regeneration

Human keratinocytes can be clonogenic or non-clonogenic. Clonogenic keratinocytes are endowed with an impressive proliferative potential, are located in the epidermal basal layer and consist of stem cells and transient-amplifying (TA) progenitors, eventually generating terminally differentiated cells. Non-clonogenic, terminally differentiated keratinocytes have lost their proliferative capacity and are located in the suprabasal layers of the epidermis (Watt 2002; Gambardella and Barrandon 2003; Blanpain and Fuchs 2006, 2014).

Barrandon and Green (1987) have identified three types of human epidermal clonogenic keratinocytes, which give rise to clones referred to as holoclones, meroclones, and paraclones. They can be isolated both from a tissue biopsy and a keratinocyte culture (De Rosa et al. 2020). Initially described in the skin, they were found also in other stratified epithelia, such as cornea (Pellegrini et al. 1999a), urethra, and oral mucosa (Corradini et al. 2016; Sceberras et al. 2020). All clonal types are endowed with proliferative capacity. But while paraclones can undergo a limited (up to 15) number of cell doublings, holoclones and meroclones can produce dozens of population doublings (Rochat et al. 1994; Mathor et al. 1996; Pellegrini et al. 1999a; Dellambra et al. 2000). The onset of replicative senescence is determined by clonal conversion, namely, progressive decline in the proportion of holoclones and meroclones and progressive increase of paraclones, the latter generating only aborted colonies (Barrandon and Green 1987; Rochat et al. 1994; Mathor et al. 1996; Pellegrini et al. 1999a; Dellambra et al. 2000; Ronfard et al. 2000; De Rosa et al. 2019). Cultures of human keratinocytes contain all clonal types and generate epithelial grafts that have been extensively used to treat massive full-thickness skin burns and to restore visual acuity in patients with chemical burn-dependent limbal stem cell deficiency (Gallico et al. 1984; Pellegrini et al. 1997, 1999b; Rama et al. 2010).

Thorough analysis of data accumulated during over 30 years of clinical application of such...
cultures, has provided compelling, yet indirect, evidence that holoclones and meroclones/paraclones are generated by keratinocyte stem cells and TA progenitors, respectively (Pellegrini et al. 1999a,b, 2013; Ronfard et al. 2000; De Luca et al. 2006, 2019; Rama et al. 2010). But formal evidence of holoclone-forming cells being authentic, self-renewing keratinocyte stem cells was gained only recently (Hirsch et al. 2017). Using proviruses as clonal genetic marks, clonal tracing of the newly formed transgenic epithelium has unambiguously shown that holoclone-forming cells are necessary and sufficient to sustain the regenerated epithelium. They continuously generate meroclones and paraclones that, as expected from TA progenitors, are short-lived and, although instrumental for proper tissue regeneration and wound healing, are progressively lost during epidermal renewal. In a nutshell, clonal tracing has shown that the main feature distinguishing the holoclone-forming cell from the other keratinocyte clonal types is its self-renewal and long-term regenerative capacity (Hirsch et al. 2017).

It follows that the essential feature of any cultured epithelial graft is the presence of an adequate number of holoclone-forming cells (De Luca et al. 2006, 2019). While paraclones could be identified based on their morphology (small irregular colonies containing large and flattened cells), holoclones and meroclones cannot be distinguished based on their growth rate and behavior and/or their shape and size (Barrandon and Green 1987; Enzo et al. 2021). Thus, a colony forming efficiency assay—measuring the number (and the shape) of colonies generated by the clonogenic cells contained in a given culture—by no means would allow to evaluate the number of holoclone-forming cells harboring a cultured epithelial graft. Such numbers can be obtained by a formal clonal analysis (Barrandon and Green 1987; Rochat et al. 1994; Pellegrini et al. 2001; De Rosa et al. 2019; Enzo et al. 2021).

**Molecular Characterization of Holoclone-Forming Cells**

Fundamental insights into stem cells of interfollicular epidermis and hair follicle have been gathered from murine studies (Fuchs 1998; Watt 2002; Blanpain and Fuchs 2006, 2014), but not all murine findings apply to humans. For instance, the murine epidermis does not contain the same types of clonogenic keratinocytes found in the human skin. Nevertheless, an important step toward molecular definition of human epidermal holoclones came from the discovery of p63 (Yang et al. 1998) as a key transcription factor sustaining murine squamous epithelia (Mills et al. 1999; Yang et al. 1999).

p63 belongs to a family that includes two structurally related proteins, p53 and p73. Whereas p53 plays a well-established role in tumor suppression, p63 and p73 play unique roles in morphogenesis. In particular, p63 null mice have major defects in their limb and craniofacial development, as well as a striking absence of stratified epithelia (Mills et al. 1999; Yang et al. 1999). This phenotype could be explained by either inability of the p63 null ectoderm to develop into epithelial lineages (Mills et al. 1999) and/or by lack of stem cell character necessary to sustain epithelial morphogenesis and renewal (Yang et al. 1999). Subsequently, it has been shown that ΔNp63α, a specific p63 isoform, underpins the proliferative, regenerative capacity of mammalian epithelial stem cells (Senoo et al. 2007). In humans, ΔNp63α is highly expressed by epidermal and limbal holoclones and it progressively declines during keratinocyte clonal conversion (Fig. 3; Pellegrini et al. 2001; Di Iorio et al. 2005). Quantification of ΔNp63αbright cells has been used as a pretransplantation assay to evaluate the number of holoclones contained in a limbal/corneal culture. Strikingly, permanent restoration of a functional corneal epithelium in patients receiving limbal cultures for the treatment of severe chemical burns requires a defined number of ΔNp63αbright holoclone-forming cells in the culture (Rama et al. 2010; Pellegrini et al. 2013). This assay, however, has not been similarly validated for epidermal cultures.

YAP is a transcriptional coactivator driving cell proliferation in many types of stem and progenitor cells and a key regulator of mechanotransduction (Zhao et al. 2008, 2011; Pan 2010; Dupont et al. 2011; Piccolo et al. 2013; Irvine and Harvey 2015). Unphosphorylated YAP translo-
cates to the nucleus, where it induces target genes through interaction with TEAD transcription factors (Zhao et al. 2008). Phosphorylation of YAP by Mst1/2 and Lats1/2 kinases in defined serine residues results in their sequestration, hence, functional inactivation, into the cytoplasm by 14-3-3 proteins (Zhao et al. 2007). YAP interacts with ΔNp63α in sustaining self-renewal and proliferative/regenerative capacity of holoclone-forming cells (De Rosa et al. 2019). The transcriptomic profile of single human keratinocytes unveiled that FOXM1, a transcription factor member of the forkhead box family, acts downstream of YAP (Enzo et al. 2021).

Nuclear YAP and FOXM1 are highly expressed in epidermal holoclones but virtually undetectable in meroclones and paraclones (Fig. 3; De Rosa et al. 2019; Enzo et al. 2021). In contrast, phosphorylated-YAP and 14-3-3σ (which is responsible for cytoplasmic sequestration of YAP), are barely detectable in holoclones and progressively increase during clonal conversion (Fig. 3; Dellambra et al. 2000; De Rosa et al. 2019; Enzo et al. 2021). Accordingly, the ablation of either YAP or FOXM1 induces the selective disappearance of holoclones (De Rosa et al. 2019; Enzo et al. 2021), while enforced YAP or FOXM1 or ablation of 14-3-3σ halt clonal conversion and sustain holoclone-forming cells indefinitely (Dellambra et al. 2000; De Rosa et al. 2019; Enzo et al. 2021).

Noteworthy, JEB keratinocytes initiate colonies with low efficiency and JEB cultures are often depleted of detectable holoclones, making ex vivo gene therapy particularly cumbersome, sometimes not doable (Dellambra et al. 1998; Mavilio et al. 2006). These features are particularly evident in JEB adults and are not observed in cultures initiated from other forms of EB (De Rosa et al. 2020). It soon became evident that the interaction of laminin 332 with integrins α6β4 is required for YAP nuclear translocation and its cotranscriptional activity (De Rosa et al. 2019). Accordingly, the expression of basal epidermal integrins decreases during clonal conversion (Fig. 3).

Both microarray and single-cell RNA-seq data have also shown that holoclone-forming...
cells display other common stem cell features, such as genes regulating DNA repair, chromosome segregation, spindle organization, and telomerase activity, and are enriched in genes regulating microtubules and actin polymerization (Enzo et al. 2021). In fact, actin bundles are distributed radially in holoclones and circumferentially in paraclones (Nanba et al. 2013). These actin filament dynamics are governed by Rac1 and are instrumental in controlling clonal conversion, hence, stem cell maintenance (Nanba et al. 2013).

Although a human holoclone molecular signature is thus emerging, the most trustworthy assay able to define the clonal composition of an epidermal culture is still the clonal analysis (Enzo et al. 2022). Further development of single-cell genetic and epigenetic analyses should give more insights that could allow to prospectively distinguish epidermal holoclones from the other clonal types.

CONCLUSION

The current management for EB patients envisages different strategies, each aiming at alleviating the clinical manifestations. The increasing number of clinical trials assessing innovative, advanced molecular therapies resurges new hopes to definitively tackle this devastating disease. But none of these advanced approaches have yet made it to a routine therapy. The genetic and phenotypic EB heterogeneity (and the ambitiousness of a regenerative medicine approach) would require the convergence of multiple expertise and disciplines, including stem cell biology, developmental and molecular biology, genetics, tissue engineering, and, not to say, a deep knowledge of all the clinical and surgical features of the different forms of the disease. Hence, a multidisciplinary collaborative effort is critical. The complexity of the disease implies that there will not be a single strategy that suits all patients. Hopefully, at the end of this steep stairway, we, together, could define a combination of truly successful therapies, most likely personalized for each EB variant, to give EB patients relief and a better life. In an ideal world, the choice of such a combination should be made independently of their own favorite strategy (and personal interest) and should not be hampered solely by its cost.

COMPETING INTEREST STATEMENT

Michele De Luca is a cofounder and member of the Board of Directors of Holostem Terapie Avanzate, S.r.l., Modena, Italy and consultant for J-TEC, Japan Tissue Engineering, Ltd.

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