Chapter

Therapy Development for Epidermolysis Bullosa

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Abstract

Although rare genodermatoses such as Epidermolysis bullosa have received more attention over the last years, no approved treatment options targeting causal mutations are currently available. Still, such diseases can be devastating, in some cases even associated with life-threatening secondary manifestations. Therefore, developing treatments that target disease-associated complications along with causal therapies remains the focus of current research efforts, in order to increase patient's quality of life and potentially their life expectancy. Epidermolysis bullosa is a genodermatosis that is caused by mutations in either one of 16 genes, predominantly encoding structural components of the skin and mucosal epithelia that are crucial to give these barrier organs physical and mechanical resilience to stress. The genetic heterogeneity of the disease is recapitulated in the high variability of phenotypic expressivity observed, ranging from minor and localized blistering to generalized erosions and wound chronification, rendering certain subtypes a systemic disease that is complicated by a plethora of secondary manifestations. During the last decades, several studies have focused on developing treatments for EB patients and significant progress has been made, as reflected by numerous publications, patents, and registered trials available. Overall, strategies range from causal to symptom-relieving approaches, and include gene, RNA and cell therapies, as well as drug developments based on biologics and small molecules. In this chapter, we highlight the most recent and promising approaches that are currently being investigated in order to provide effective treatments for patients with epidermolysis bullosa in the future.

Keywords: Epidermolysis bullosa, genodermatoses, gene therapy, drug development, wound healing, pruritus, fibrosis, squamous cell carcinoma

1. Introduction

Epidermolysis bullosa (EB) refers to a group of rare genodermatoses typically characterized by vulnerability of the skin to friction or trauma, leading to blistering and wounding to various extents, *i.e.* from localized and mild to generalized and severe, depending on the respective subtype of the disease. EB subtypes are classified according to the mutated gene, the resulting product of which is either functionally impaired or absent, and the level at which tissue cleavage occurs. While involvement in some subtypes are restricted to the skin, for others, extracutaneous organs (*i.e.* mucosa and eyes) may be involved, along with a multitude of secondary manifestations that significantly impair patients' life quality (QoL) and may

even be life-threatening. Among these is the development of an aggressive form of cutaneous squamous cell carcinoma (cSCC) in patients with the severe dystrophic subtype of the disease [1–3].

Worldwide approximately 500,000 people suffer from EB, which can be classified into four major groups [1]. Mainly dominantly inherited mutations within genes encoding keratin 5, 14 and plectin lead to EB simplex (EBS), associated with intraepidermal blister formation (**Figure 1**). Mutations within genes, encoding laminin-332, type XVII collagen or integrin-α6β4, are the main causes of the junctional form of EB (JEB), characterized by tissue separation within the lamina lucida of the basement membrane zone (BMZ). The severe dystrophic variant of EB (DEB) is caused exclusively by mutations within the gene *COL7A1*, encoding type VII collagen, resulting in tissue separation within the sub-lamina densa (**Figure 1**). Kindler syndrome is caused by recessive mutations within the *KIND1* gene leading to a complete loss of encoded Kindlin-1 and blistering in multiple skin layers [1].

Despite advances in our understanding of the spectrum of pathologies associated with the different subtypes of EB, a systemic cure is still out of reach. However, the increasing number of trials that are being conducted reflects significant progress in clinical research, including strategies to correct and/or modulate the aberrant molecules and mechanisms underlying this devastating disease. [4–7] The remarkable differences between EB-types and numerous subtypes, renders the development of therapies a complex challenge, as both inter-subtype and inter-individual differences require the development of more personalized treatments.

Major complications in EB range from itch and pain, to a predisposition to wound chronification and tumor development, with molecular contributors deriving from various sources including different tissue-associated cell types, matrix components, as well as inflammatory events. The majority of these comorbidities, in and of themselves, are not unique to EB, so that the repurposing of clinically approved treatments against such symptoms represents an attractive path for a more rapid market approval of these compounds for EB. Furthermore, new

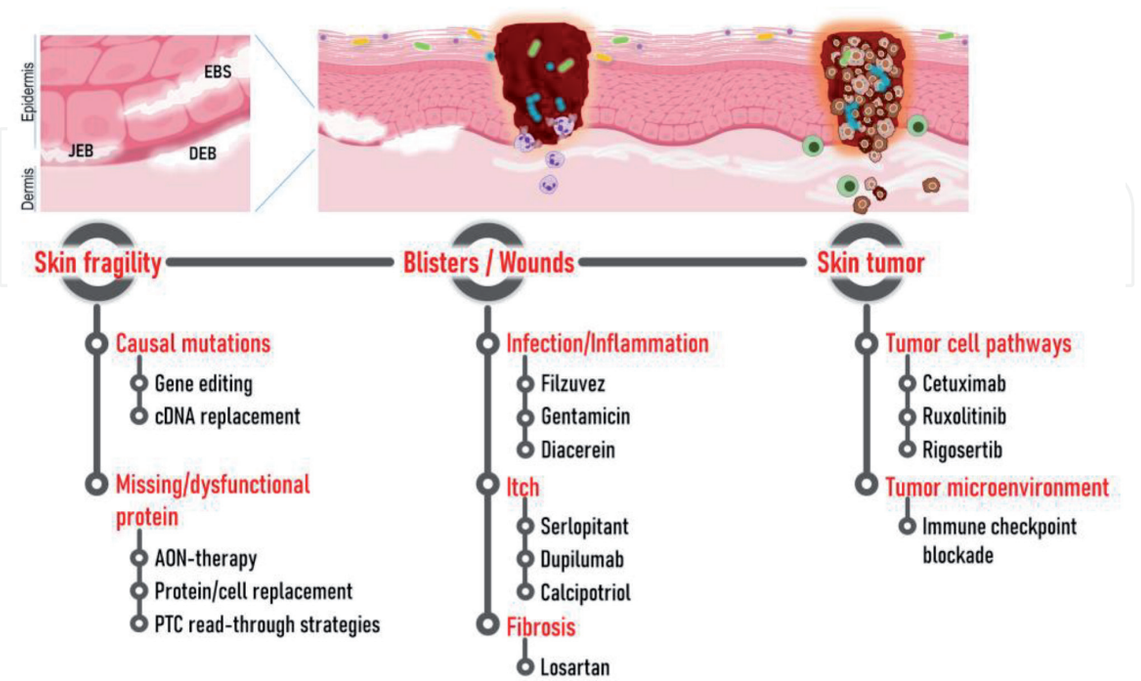

Figure 1.
Therapeutic targets in EB and strategies in development against them. EB is a rare hereditary skin fragility disorder characterized by blistering and wounding. In severe subtypes of the disease, wounds degenerate into tumors. The different therapeutic strategies to target important aspects of the disease are depicted.

evidence arising in the study of these common conditions can be leveraged to direct therapy development in EB. Nevertheless, rigorous evaluation for safety in this specific, vulnerable patient group is warranted for any candidate therapeutic. This is especially true for cancer therapies which are associated with significant cellular toxicity and often have the adverse effect of exacerbating the wound healing deficiencies associated with the disease.

2. Therapy development for EB

2.1 Causal therapies for epidermolysis bullosa

2.1.1 Gene replacement therapies for epidermolysis bullosa

The development of causal therapies has always been a main focus of EB research (**Table 1**).

Currently, gene replacement strategies that exploit viral vectors to introduce full-length wild type cDNA copies of the affected gene into the skin cells of patients [14], have advanced the furthest in clinical trials. However, this strategy relies on genomic integration of the transgene to achieve long-term restoration of gene

EB subtype	Therapeutic goal	Therapeutic strategy	Targeted gene/protein	Status	Ref
EB simplex	Causal therapy	Gene editing	*KRT5, KRT14*	pre-clinical	[8, 9]
		SMaRT	*KRT14, PLEC1*	pre-clinical	[10–13]
Junctional EB	Causal therapy	cDNA replacement	*LAMB3*	clinical	[14–17]
		Gene editing	*LAMB3*	pre-clinical	[18]
	Read-through therapy	PTC read-through	*LAMB3*	clinical	[7]
Recessive dystrophic EB	Causal therapy	cDNA replacement	*COL7A1*	clinical	[19–23]
		AON	*COL7A1*	pre-clinical	[24–27]
		Gene editing	*COL7A1*	pre-clinical	[24, 28–36]
		SMaRT	*COL7A1*	pre-clinical	[10, 37–40]
	Protein therapy	Protein replacement	Type VII vollagen	pre-clinical	[41]
	Cell therapy	Allogeneic fibroblast injection	Type VII collagen	clinical	[42]
	Read-through therapy	PTC read-through	*COL7A1*	clinical	[43]

Table 1.
Overview on causal therapies in epidermolysis bullosa and their current clinical status.

function, which bears a low risk of genomic toxicity due to insertional mutagenesis that can result in tumor development as shown for for X-linked severe combined immunodeficiency (X-SCID) [44]. However, no such deleterious events have been observed thus far for EB [14–17, 19]. In general, cutaneous gene therapies have the advantage that grafted skin areas are easy to monitor, with developing tumors easily detected and promptly excised. Until now, transplantation of genetically corrected skin grafts represents the most auspicious approach, due to the limited number of viral vectors suitable for *in vivo* targeting. Poor transcutaneous delivery of the vector and the size of the transgene represent further limitations [45, 46].

To date, the most successful application of gene replacement therapy has been achieved in junctional EB (JEB) patients carrying mutations in the *LAMB3* gene [14, 16, 17]. In all three cases, epidermal stem cells isolated from skin biopsies and expanded *in vitro*, were treated with a Moloney leukemia virus (MLV)-derived retroviral vector expressing the full-length cDNA of *LAMB3*. Treated cells were then expanded into epidermal sheets, which were transplanted back onto the patient. To this day, the first patient treated in 2006 still retains the transgenic epidermis, which at 6.5 years follow-up, appeared normal, blister-free, and showed accurate localized expression of laminin-332 within the skin, and no adverse effects reported thus far [15]. More recently, the same procedure was applied to treat an eight-year old JEB patient with life-threatening skin loss due to a bacterial infection. Over the course of successive treatments, up to ~80% of the patient's skin was surgically replaced by genetically corrected skin, demonstrating the life-saving potential of this therapeutic strategy for genodermatoses. Genetic analyses of the skin grafts clearly demonstrated that the transgenic epidermis was sustained by a defined number of epidermal stem cells with long-term regenerative potential [17].

Despite these successes, attempts to apply the same strategy in recessive dystrophic EB (RDEB) demonstrated no long-lasting effects. While long-term *COL7A1* expression could be attained following treatment, variable clinical outcomes were observed, with persistent type VII collagen expression detected in only two out of seven treated patients at two years post transplantation. [19, 20]

For most patients, improved wound healing and an accurate deposition of type VII collagen within the regenerated skin could be detected. However, expression of the transgene significantly decreased over time [19]. Possible reasons for this include the size of the *COL7A1* cDNA (~9 kb), the random nature of viral integrations, or post-transcriptional deregulation via aberrant splicing [21, 47]. In this respect, targeting *LAMB3* via a gene replacement therapy bears a big advantage over *COL7A1*, as the phenotype of JEB is associated with a significant depletion of epidermal stem cells [48]. Here, the YAP/TAZ mechano-sensing pathway plays a major role in sustaining holoclones downstream of Laminin-332 signaling. Holoclones represent stem cells with the greatest reproductive capacity and are essential for epidermal regeneration. Thus laminin-332 gene therapy likely rescues YAP activity, enabling the maintenance of the pool of epidermal stem cells [48]. Thus, while these clinical trials in JEB and DEB demonstrate the potential of gene replacement-based therapies in EB, they also reveal the varying therapeutic efficiencies that can be expected among the different EB types, potentially depending on the biology of the respective matrix protein affected.

In contrast to targeting patient keratinocytes, a combined gene and cell therapy approach using patient autologous fibroblasts was recently evaluated in a phase I, open-label, single-center clinical trial in four RDEB patients [22]. Based on previous preclinical data [23], patient fibroblasts were first modified *ex vivo* to carry a full-length codon-optimized *COL7A1* cDNA using a self-inactivating lentiviral vector. The gene-modified autologous fibroblasts were then injected intradermally back into the patients. The treatment was well-tolerated and no serious side effects

were observed. Mean fluorescence intensity of type VII collagen staining in treated skin was 1.26-fold to 26.10-fold increased over non-injected skin, with enhanced expression sustained for up to 12 months in 2 out of the 4 patients [22]. This study demonstrated the potential of a combined gene & cell therapy approach for the treatment of RDEB, although more clinical data is required to evaluate the benefit for the patient.

2.1.2 Gene editing strategies for epidermolysis bullosa

Gene editing platforms based on programmable nucleases have advanced at a rapid pace, such that the correction of any gene is now, at least in theory, conceivable. At their core, designer nucleases consist of DNA endonucleases, such as zinc-finger nucleases (ZNF), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR) /CRISPR-associated protein 9 (Cas9), which are guided to specific DNA loci of interest, where they generate double-strand breaks (DSBs) and trigger the activation of DNA repair mechanisms [49, 50]. The most frequent repair pathway, termed non-homologous end joining pathway (NHEJ), relies on the introduction of small insertions and deletions (indels) at the DSB site [51], which can be leveraged for the inactivation of genes carrying dominant negative missense mutations [8, 52], or for the reframing of genes bearing pathogenic frameshift mutations [28, 29].

While not a genetic correction *per se*, gene disruption can be a suitable way of targeting dominant alleles via causing the coding sequence to run into a premature termination codon (PTC) during translation. Ribosomal stalling and activation of the nonsense-mediated mRNA decay pathway triggers the degradation of the edited mutant transcripts [53]. We have exploited this strategy to disrupt a dominant negative *KRT5* mutation whilst leaving the wild type allele intact [8]. This targeting strategy should be applicable to a broad number of EBS patients. Similarly, for DDEB, Shinkuma et al. applied an allele-specific gene disruption approach to target a dominant negative mutation within *COL7A1* [30].

The same strategy can also be used to reframe mutant mRNA and was recently shown by several groups to be a promising editing approach in RDEB [28, 29, 31, 32]. Leveraging recently developed algorithms to accurately predict end-joining (EJ) repair outcomes following CRISPR/Cas9-mediated cleavage, we were able to achieve significant restoration of *COL7A1* function after targeting a pathogenic frameshift mutation within exon 73 of *COL7A1* [29]. Sequence composition around the Cas9 binding site predicted a single adenine sense-strand insertion at the *COL7A1* target locus as the dominant EJ repair outcome, which would restore the reading frame of the message while introducing a single amino acid change in the protein. Indeed, we detected this precise nucleotide modification in 17% of all next generation sequencing (NGS)-analyzed *COL7A1* alleles, following a single RNP treatment. In all gene-edited cells analyzed, > 70% exhibited restored functional protein expression, underscoring the potential of end-joining based DNA repair strategies for restoring gene function in EB [29].

Of course, the holy grail of gene therapy has been to achieve a traceless repair of the disease-causing mutation. With current editing technologies, this is now attainable by invoking the high-fidelity homology directed repair (HDR) pathway. By providing an exogenous HDR donor sequence, which bears homology to the target region, the exchange of whole gene regions or individual nucleotides can be achieved [18, 24, 49, 50]. However, in comparison to EJ-based targeting strategies, gene editing efficiency is generally reduced, as homologous recombination is only active during the late S/G2 phase of the cell cycle [54]. Nevertheless, in EB, HDR-based gene repair strategies have been successfully applied to EBS [9] and

RDEB cells [24, 33, 35] *in vitro*. The focus of therapy development currently lies on improving safety and efficiency, which are both prerequisites for any future *in vivo* application of this strategy. Towards this end, to circumvent the known off-target activity of wild type *Streptococcus pyogenes* Cas9 (SpCas9), we utilized a mutant Cas9D10A nuclease, which predominantly induces single-strand nicks instead of double-strand breaks within the DNA [55, 56]. Indeed, we found that targeting of mutant *KRT14* or *COL7A1* alleles with two guided nickases in a double-nicking configuration was safer and more efficient than the use of wild type spCas9 [9, 24]. Additional efficiency can be gained by optimizing both, the format and the delivery of the gene-editing molecules. Currently, electroporation of *COL7A1*-specific RNPs together with single-stranded oligonucleotide HDR templates have resulted in the highest repair efficiencies [34].

The application of HDR-based approaches to the correction of patient-derived induced pluripotent stem cells (iPSCs) further increases the range of therapeutic options for patients, especially as the isolation of epidermal holoclones can be a limiting factor [14, 34]. Pre-clinical studies using corrected iPSCs, that were then differentiated into keratinocytes and fibroblasts, and used to generate three-dimensional skin equivalents (HSEs) on the backs of immunodeficient mice, showed normal type VII collagen expression and restored anchoring fibrils [34]. Alternative strategies, not based on homology-directed repair, comprise base editing, which has proven to be a suitable option for correcting pathogenic mutations in RDEB [36]. The most recent genome editing tool, prime editing, can be used to directly write new genetic information into a selected genomic locus using a Cas9 nickase fused to an engineered reverse transcriptase (RT) domain [57]. Via a prime editing guide RNA (pegRNA), which specifies the target site and represents the RT template encoding the desired edit, the prime editor is directed to the target locus. Here, the Cas9 nickase makes a single strand DNA break, that induces the hybridization of the nicked genomic strand to the complementary primer binding site (PBS) sequence, located within the pegRNA. A subsequent reverse transcription of the RT template, carrying the desired edit, followed by a cellular DNA repair mechanism lead to the insertion of the respective genetic modification at the target site [57]. Prime editing potentially improves safety, efficiency and applicability, and its application to EB will undoubtedly be confirmed in appropriate disease models in the near future.

2.1.3 RNA-based therapies for epidermolysis bullosa

A promising RNA-based strategy for the restoration of functional protein expression in EB is based on the use of antisense oligonucleotides (AON) for the specific knockdown of genes or their modification via splicing interference [58]. AONs are generally short fragments of modified DNA or RNA which, in the case of splicing modulation, hybridize to splicing elements (*e.g.* splice acceptor site or enhancers) within an in-frame target exon during pre-mRNA splicing, thereby masking it from the splicing machinery and resulting in its exclusion from the mature transcript. Thus, a truncated protein carrying an in-frame deletion of one or more exons is translated from the new transcript. In the last decade, AON-mediated splicing modulation has been successfully applied in DEB keratinocytes to skip in-frame *COL7A1* exon 70 [25], exon 73 [24, 26], exon 80 [26], and exon 105 [27] that carried dominant or recessive disease-causing mutations. The resulting proteins, though shorter, retained functionality. Indeed, *COL7A1* is amenable to AON-based exon skipping strategies because of the numerous short in-frame exons that encode its collagenous domains [24]. Beyond proof-of-concept, formulation of an exon 73-specific AON, named QR-313, within a carbomer-composed gel for topical treatment of wounds in DEB, led to enhanced type VII collagen levels in human RDEB

skin [24], and is currently being evaluated for safety and efficacy in clinical trials (NCT03605069). However, not all exons are suitable targets for exon skipping. Furthermore, amino acid sequences potentially vital for protein function may be deleted. Thus, AON-based gene repair approaches need to be carefully evaluated for each targeted gene, exon and even mutation, prior to their clinical application [27].

Another RNA-based strategy for mRNA correction, namely RNA *trans*-splicing (also termed spliceosome-mediated RNA *trans*-splicing, SMaRT), has emerged as an attractive therapeutic option for the correction of EB-associated mutations on the RNA level [10–12]. Here, the endogenous splicing machinery is exploited to selectively exchange mutation-bearing regions of the target pre-mRNA, with the corresponding wild type sequence from an exogenously provided RNA *trans*-splicing molecule (RTM) [59]. The engineered RTM contains a binding domain to direct its hybridization to the target pre-mRNA, as well as splicing elements required for efficient splicing, in addition to the wild-type coding sequence to be restored [59]. This approach has been applied to accurately restore gene function in a variety of human genetic diseases, including hemophilia A [60], muscular dystrophy [61, 62] or Alzheimer's disease [63]. In EB, SMaRT-mediated RNA editing was first achieved for the *PLEC* gene [12], but has more recently, been used to correct the EB-associated genes *KRT14* [10, 11, 13] and *COL7A1* [37–40] *in vitro* and in pre-clinical animal models. However, while these studies indicated a potential clinical applicability of SMaRT technology in gene therapy for EB, the efficiency of this RNA-based method needs to be significantly improved if it is to move forward towards clinical translation.

2.1.4 Protein- and cell-based therapies for epidermolysis bullosa

Protein replacement strategies were recently applied in preclinical studies for RDEB. The local or intravenous injection of recombinant type VII collagen led to its homing to the dermal-epidermal junction and promoted wound healing [41]. Another therapeutic option for RDEB is the administration of type VII collagen-expressing allogeneic fibroblasts into healing RDEB wounds. Particularly when injected intradermally allogeneic fibroblast therapy resulted in a significant decrease in wound area when compared to standard of care after 2 and 12 weeks of treatment [42].

2.1.5 Read-through strategies for epidermolysis bullosa

Premature stop codon (PTC) read-through strategies rely on agents that allow for the incorporation of a random amino acid at the PTC position in the mRNA. Depending on the importance of the original amino acid to protein function, as well as impact of the introduced amino acid to *e.g.* protein folding, stability, and post-translational processing, PTC read-through therapies can result in the synthesis of a functional full-length protein. This strategy has been shown to be feasible in RDEB-derived cells [64, 65]. Cogan et al. treated RDEB keratinocyte cell lines and RDEB fibroblasts carrying PTC mutations, with the aminoglycosides geneticin, gentamicin and paromomycin. Full-length type VII collagen was accurately synthesized and secreted in a dose-dependent and sustained manner, highlighting the therapeutic potential of PTC read-through approaches for RDEB patients [65]. This resulted in a clinical trial to assess safety and efficacy of topical and intradermal gentamicin treatment in 5 RDEB patients with nonsense mutations application led to induced type VII collagen expression and anchoring fibril generation at the dermal-epidermal junction of treated skin areas, with improved wound closure and reduced blistering [43]. However, the toxicity associated with long-term aminoglycoside use currently hinders their widespread clinical application for these purposes. Alternatively, the FDA-approved anti-inflammatory drug amlexanox has

been demonstrated to induce full-length collagen type VII expression *in vitro* in 8 out of 12 different RDEB PTC alleles tested. Furthermore, read-through synthesis correlated with the phosphorylation of the RNA helicase UPF-1, suggesting that inhibition of nonsense mediated decay of the PTC-containing mRNA contributed to its mechanism of action [64]. However, increased read-through translation alone is insufficient to achieve proper function, and additionally accurate deposition of the protein at the basement membrane zone likely needs to be confirmed for each PTC mutation. Similar to the mentioned RDEB studies, the aminoglycoside gentamicin was recently applied to JEB keratinocytes carrying various nonsense mutations within the *LAMB3* gene [66]. As a result, the authors achieved PTC read-through leading to the synthesis and secretion of the respective laminin chain protein as well as the restoration of laminin-332 assembly [66]. Nevertheless, future studies are required to address current issues concerning read-through-based approaches such as the toxicity and bioavailability of applied compounds and their interactions within treated cells and organisms.

In summary, numerous strategies to target the genetic cause of EB, as well as ameliorate disease-associated complications, are under intensive investigation. These act at various levels, from genes and gene products, to cellular pathways, tissue processes, and systemic events. While each strategy has distinct strengths and challenges, they all share the overarching aim of significatnly improving the QoL of patients (**Figure 2**).

2.1.6 Immunological aspects of causal therapy

Immunological tolerance to self-antigens result from central and peripheral tolerance mechanisms. Central tolerance in the thymus results in either negative selection of self-reactive T cells or development of self-specific suppressive regulatory T cells, both of which require expression and presentation of self-antigens to developing thymocytes. Additionally, various peripheral mechanisms of tolerance protect the body from deleterious reactions against self-tissues. These include anatomical sequestration of self-antigens, deletion of peripheral autoreactive lymphocytes, the development of functional unresponsiveness of lymphocytes (anergy) and action of regulatory T cells [67, 68].

A major risk in patients, especially those completely lacking expression of the affected protein, is that this protein is missing from the repertoire of self-antigens presented during central tolerance establishment. As the aim of causal therapies is to restore the missing protein or repair a defective (*e.g.* truncated) one, these include an inherent risk of inducing adverse immune reactivity against the restored wild type protein, at least parts of which would be recognized in patients as foreign. For these reasons, only patients with residual expression of the EB-associated protein have been included in gene therapy trials to date [14, 16, 17, 19]. Despite this, transient circulating antibodies reactive against the gene-correction product, and also tissue-bound antibodies deposited within the graft, were reported in some participants [19, 20]. However, these did not correlate with rejection of the graft. In addition, a phenomenon called epitope spreading could theoretically occur in patients that mount an immune reaction against the gene-correction product, leading to autoimmunity against the endogenous mutated/residual protein present throughout the body's epithelial tissues. This systemic immune reaction would likely manifest as blistering within the graft, as well as worsened blistering distal to the graft. Thus, monitoring immunological parameters (*e.g.* specific antibody formation) and diffuse new-onset blistering is warranted in study participants. While detailed immunological studies are still largely lacking, the results suggest ex vivo gene-replacement therapy to be a safe therapeutic approach in patients who lack pre-existing immune reactivity and express residual protein. However, future

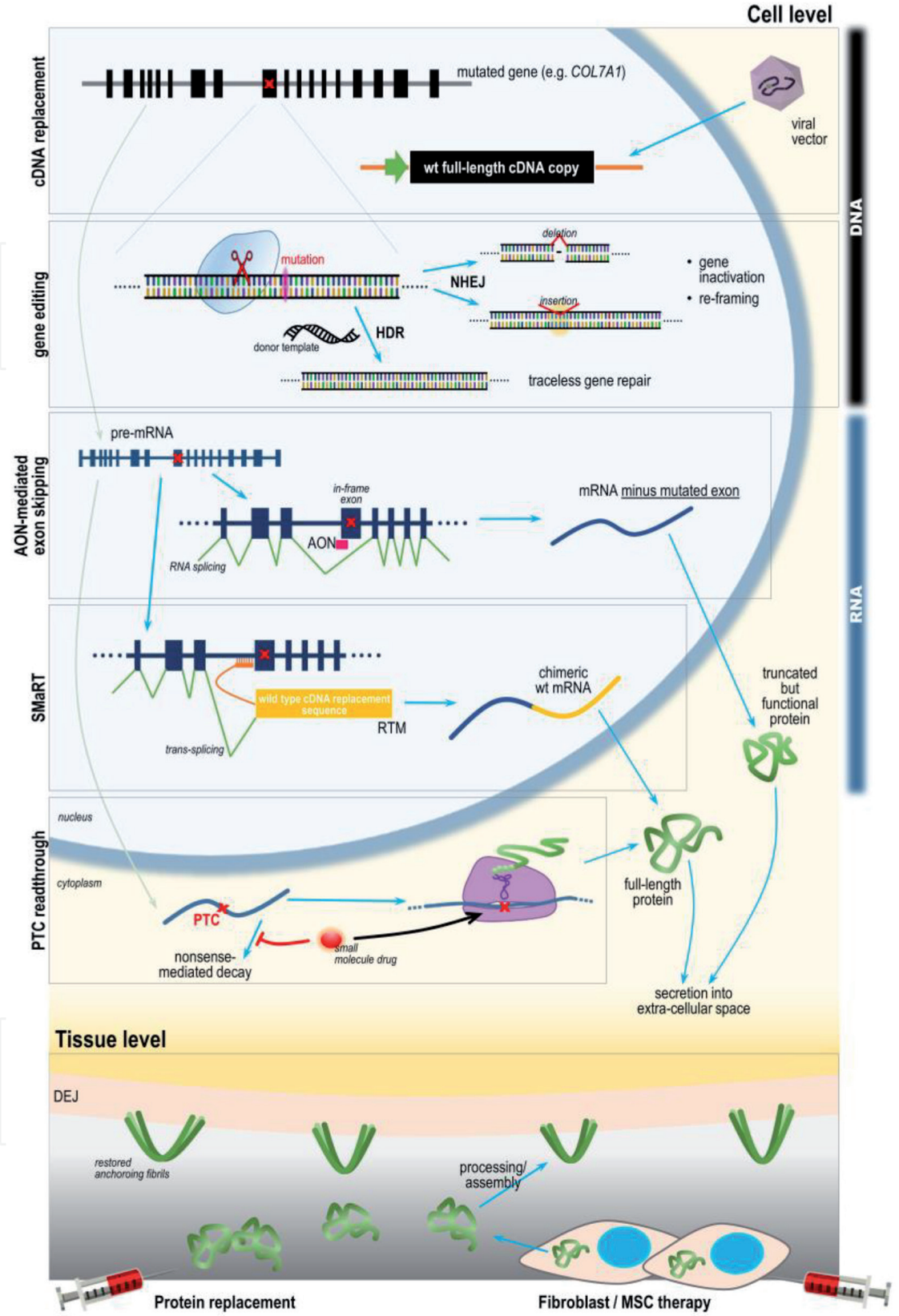

Figure 2.
Causal therapy options for epidermolysis bullosa. Current strategies targeting the genetic cause of EB can be designed to act either on DNA level (cDNA replacement, gene editing), RNA level (AON-mediated exon skipping, SMaRT), RNA/protein level (PTC readthrough) or tissue level (protein replacement fibroblast/ MSC therapy).

trials should aim to also include patients without any residual protein expression, who oftentimes display a more severe phenotype, and where there is likely no pre-existing tolerance to prevent adverse immune reactions against the gene-correction

product. Towards this goal, further research is required to exploit peripheral immune tolerance mechanisms to control the response to neo-antigens in the skin.

2.2 Treating complications of EB

While gene therapy is the only curative option for EB, strategies to ameliorate symptoms are critically needed to increase patient's QoL and prevent severe complications of the disease until causal therapies are available for all EB patients. The number of preclinical and clinical studies published, including those currently registered, reflects the great effort placed into providing such. Strategies to identify suitable candidates are diverse, but great potential lies in drug repurposing, as this facilitates timely development of potent treatments by leveraging already existing pre-clinical and clinical data. Even so, the methodological challenges inherent to conducting studies in rare disease populations can complicate the clinical evaluation of repositioning such drugs for EB [69].

The active components of drugs generally comprise small molecules or biologics. While low molecular weight small molecules can be derived chemically and exhibit distinct advantages regarding delivery and route of administration, biologics, which are much larger, often interfere very specifically with distinct pathomechanisms and show overall less toxicity. Functionally, small molecules are frequently designed as inhibitors of *e.g.* enzymes, whereas biologics usually have a specific active function (*e.g.* antibodies, enzymes, nucleic acids).

In the context of EB several approaches addressing various complications have been reported, with the majority of primary outcomes measured being improvement of wound healing, reduction of blistering, and mitigation of itch. While some of the evaluated compounds have reached late stage clinical trials, first marketing approvals are still awaited [4–7, 69].

2.2.1 Reduction of blistering

Across the various EB types, blistering of the skin may be the first clinical manifestation of skin fragility following mechanical friction or trauma. While in some patients blisters heal without scarring, in patients suffering from more severe subtypes these degenerate into wounds and are accompanied by multiple comorbidities. Thus, preventing blistering or accelerating their resolution is a logical primary outcome measure for clinical trials due to its relevance to patients, at least in distinct patient cohorts [7]. Particularly in EBS, where patients only rarely develop wounds, reducing blister numbers will improve patients' QoL substantially. Especially during childhood, EBS patients are prone to developing numerous blisters, which may prevent children from *e.g.* learning to walk, or playing with other children. However, also for dystrophic and junctional EB clinical studies evaluating a treatment's impact on blister numbers have been published [7].

For EBS, uncovering the pathways and molecular mediators underscoring the pathogenic keratin biology in cells, proved instrumental to the development of a topical formulation of the drug diacerein, which has been shown in randomized controlled trials (RCTs) to significantly reduce blister numbers in comparison to patients who received placebo [70, 71]. Moreover, during the patient follow-up period when no treatment was applied, a delayed recurrence of blisters was observed, pointing towards a long-term stabilization of the skin. Diacerein is a small molecule that interferes with the expression and signaling of the pro-inflammatory cytokine IL-1ß at various levels. Upregulation of IL-1ß, triggered by the accumulation of mutated keratins, is characteristic for distinct subtypes of EBS. However, the reciprocal effect of IL-1ß to further induce the expression of mutant

keratins was also observed in patient cells. Thus, interfering with this positive feedback loop proved beneficial to stabilizing the keratinocyte's intermediate filament network and consequently, also the patient's skin [72].

2.2.2 Wound healing

EB patients develop wounds throughout their lifetime, and their management and daily care routine represent a major burden that is accompanied by substantial discomfort. There is currently no standard treatment for the treatment of non-healing or severely infected wounds in EB. Defects in wound healing, associated with infections and persistent inflammation are presumed major drivers of wound chronification, which is a major risk factor for the development of particularly aggressive squamous cell carcinomas (SCC) [2, 3]. Thus, means to improve wound healing, and thereby prevent downstream complications that severely decrease QoL and that may even be life-threatening, are urgently needed. This is also reflected by the high number of clinical trials that primarily aim to improve wound healing, either by applying drugs that modulate wound healing associated pathways (*e.g.* modulation of inflammation), or, if feasible, by directly targeting the EB-causing gene products using drugs that induce read-through of PTCs.

Promising outcomes were recently reported from a trial using anti-inflammatory/immunomodulatory betulin-rich birch bark extract (Filzuvez, previously Oleogel S-10), wherein 41.3% of patients treated with Oleogel-S10 met the primary endpoint of target wound closure within 45 (± 7) days as compared to 28.9% of patients within the placebo arm. Furthermore, among the EB subtypes evaluated, patients with RDEB appeared to be particularly responsive to treatment (NCT03068780). In the context of wound healing, induction of PTC read-through as a means of triggering re-expression of genes harboring nonsense mutations, is particularly attractive in cases where the drugs being evaluated for these purposes have known antibacterial and anti-inflammatory activity. Particularly for junctional and dystrophic EB patients, clinical trials investigating the aminoglycoside antibiotic gentamicin are still ongoing [43, 73, 74] (NCT04140786, NCT03526159, NCT04644627, NCT03392909). Additionally for RDEB, the anti-inflammatory, anti-allergic immunomodulator Amlexanox, typically used against mouth ulcers, has emerged as a novel candidate in preclinical studies [64].

2.2.3 Pruritus

Pruritus is a particularly agonizing aspect associated with all subtypes of EB which not only impairs patients' QoL, but also leads to additional skin damage as it provokes scratching. Even though pruritus is not a life-threatening symptom *per se*, its overall influence on well-being is tremendous. In general, itch is a major problem associated with various diseases like atopic dermatitis, psoriasis, and nephrologic conditions. Yet, underlying molecular mechanisms are still not fully understood. Numerous inflammatory mediators have been associated with pathological itch, but despite pruritus being a severe clinical problem, effective treatments still represent an unmet medical need [75].

For patients with EB, a handful of studies targeting itch have been published, the most recent being a randomized-controlled trial (RCT) evaluating the neurokinin-1 receptor (NK1R) antagonist Serlopitant in patients with any EB subtype [76]. Serlopitant disrupts Substance P (SP) associated signaling by preventing its binding to NK1R. Expressed on multiple skin cell types, NK1R is thought to play a major role in the transmission of itch signals in the peripheral and central nervous systems [77]. In the RCT above, 14 EB patients received serlopitant or placebo over a period

of 8 weeks, and reduction of pruritus was assessed using a numeric rating scale. Even though results were not statistically significant, patients who had received the investigational drug tended to achieve a ≥ 3 point reduction in itch compared to placebo, and a positive impact on QoL was reported by the patients. However, a larger clinical trial will be needed to provide clear evidence on the efficacy of serlopitant in reducing pruritus in patients with EB [76].

Another agent currently being evaluated against pruritus in patients with EB-pruriginosa is the anti-interleukin-4 receptor alpha (IL-4Rα) monoclonal antibody Dupilumab [78, 79]. Already indicated for atopic dermatitis, Dupilumab inhibits both IL-4 and IL-13 signaling and modulates Th2-mediated immune mechanisms. The promising outcome of both studies, which included a total of three patients, might provide a rationale for larger RCTs in the future that extend to other subtypes of EB.

Interestingly, in a single-patient observational study aimed primarily at investigating the wound healing benefits of a low-dose topical calcipotriol ointment in DEB, a significant reduction of itch was reported as a highly patient-relevant outcome. Calcipotriol is an analogue of the active form of vitamin D3, an important skin homeostasis factor with roles in cell proliferation, differentiation, antimicrobial defense, and immune modulation. Calcipotriol has proven anti-proliferative effects in keratinocytes, which is leveraged for the treatment of plaque psoriasis. For this reason, we investigated a lower concentration with respect to antimicrobial peptide induction in DEB keratinocytes. In addition to the complete closure of a chronic wound within two weeks of treatment, we observed significant improvement in the diversity of the skin microbiota on the treated skin area, with complete clearance of *Staphylococcus aureus* by the end of the treatment [80]. The low dose ointment was evaluated in a small double-blind, placebo-controlled phase II clinical trial (EudraCT: 2016–001967-35), where a significant and steady reduction in pruritus was observed with calcipotriol treatment compared to placebo [81]. These results support conducting a follow-up trial to investigate its impact on itch in patients with EB, particularly given calcipotriol's reported anti-neoplastic effects.

2.2.4 Fibrosis

Repeated cycles of injury and subsequent persistent inflammation trigger a cascade of events leading to progressive fibrosis, followed by tissue stiffening and increased risk of tumor development in patients with dystrophic EB. Additionally, fibrotic webbing at limb extremities post-wounding ultimately leads to fusion of fingers and toes (called mitten deformities), severely limiting their use. Thus, strategies to support a normal course of wound healing are investigated to avoid or minimize deviations from deposition of a normal skin matrix [82]. A key player in EB-associated fibrosis is TGF-ß, a pro-inflammatory cytokine whose pleiotropic effects are highly context-dependent, and which has been shown to be constitutively expressed in RDEB-skin [83, 84]. While TGF-ß1 promotes wound healing under normal conditions, excessive TGF-ß1 signaling leads to abnormal ECM deposition and scar formation, as confirmed in a type VII collagen hypomorphic mouse model [85, 86]. Thus, modulating the expression of TGF-ß1 was hypothesized to be beneficial in reducing fibrosis. In this context, losartan, an angiotensin II antagonist with anti-fibrotic effects, has been evaluated in preclinical studies, where Nystrom *et al* achieved a significant reduction in fibrosis in collagen VII hypomorphic mice. This approach led to reduced TGF-ß signaling, normalized skin extracellular matrix composition, and delayed progression of mitten deformities [83]. Based on these results, a phase I/II clinical trial to evaluate the safety, tolerability and efficacy of losartan in children and adolescents with RDEB is currently underway (Eudra-CT: 2015–003670-32).

In a drug repurposing approach, endoglin (CD105), a type III co-receptor for TGF-1, and raloxifene, an estrogen receptor modulator, were tested in a pre-clinical setting for their potential to attenuate RDEB-associated fibrosis. Indeed, both drugs were shown to modulate profibrotic events, rendering them potential candidates for repositioning both compounds for the treatment of patients with EB [86].

2.3 EB-associated squamous cell carcinoma

Cutaneous tumors are a life-threatening complication that arise especially in patients with RDEB. Owing to the repeated cycles of wounding, infection and inflammation, RDEB patients are at especially high risk of developing aggressive squamous cell carcinoma (RDEB-SCC) with high risk-features. The sites of tumor occurrence are predominantly at sites of chronic and long-term wounds [87], especially on the extremities [88], indicating that tumorigenesis is related to the pathology of RDEB. The SCCs tend to arise in early adulthood, with a reported median age of 29 years at time of diagnosis, although the youngest case reported was in a 6-year old patient [89]. In comparison to the general population, RDEB patients have an estimated 70-fold higher risk of developing SCC [90], with cumulative risk rising from 7.5% at age 20 years, to 67.8% at age 35 years, to 90.1% by age 55 [2]. Despite aggressive therapy with multiple treatment modalities, median survival time from time of first diagnosis is 4–5 years [89], making RDEB-SCC the primary cause of premature death in these patients. The first choice of treatment still consists of wide local excision of the tumor, and even amputation of the extremity is sometimes necessary. Radiotherapy and conventional chemotherapeutic approaches have been mostly used palliatively in EB SCC and considering their strong adverse effects (*e.g.* desquamation of the skin upon radiotherapy) should be used carefully when applied to this vulnerable patient group [91].

Genomic analyses combined with transcriptomic profiling of tumors highlight cell endogenous mutagenic processes mediated by APOBEC enzymes, which are associated with an innate defense mechanism against ongoing microbial infection, as a major driver of carcinogenesis in RDEB. These observations indicate that effective wound management, which includes an antimicrobial component, could potentially lower cancer risk in these patients. Genetically, RDEB-SCC closely resembles ultraviolet (UV) light-induced SCC and SCC of the head and neck (HN-SCC), with driver mutations in known cancer-associated genes such as *CASP8, NOTCH1, TP53, FAT1, CDKN2A, HRAS, ARID2, and KMT2B* frequently observed [92]. While these similarities suggest that therapies proven to be efficacious in other SCCs would also be effective in RDEB SCC, careful consideration of the background pathology of EB is needed. The significant overlap in cellular processes associated with wound healing and tumor development (*e.g.* proliferation, migration, vascularisation/angiogenesis, matrix remodeling) dictates that a delicate balance needs to established wherein tumor inhibition is not interfering with wound healing, especially when long-term treatment is considered.

2.3.1 EGFR inhibition

Cetuximab, a monoclonal antibody targeting the extracellular domain of epidermal growth factor receptor (EGFR), has been used for therapy against both HN-SCC, as well as advanced unresectable cutaneous UV-SCC [93, 94]. This agent inhibits tumor cell proliferation by blocking receptor tyrosine kinase activity upstream of known survival-, growth-, and migration- signaling cascades mediated by *PI3K/AKT*, *RAS/MAPK*, and *JAK/STAT* [95, 96]. Cetuximab shows significant efficacy against EGFR-positive, wild type RAS tumors, while those bearing RAS

mutations are resistant to treatment [97]. EB-associated SCCs frequently express EGFR, but with noticeable differences in expression level which could impact responsiveness to treatment [98]. To date, only a handful of cases have reported the use of cetuximab in EB patients with advanced cutaneous SCC, with limited beneficial effects on survival [88, 94, 98–100]. Progression-free survival in seven patients reported in the literature ranged from three weeks to nine months. Better outcomes have been observed when cetuximab was given as a first line treatment suggesting that treatment might be more efficacious when administered early. Of note, wound healing deficits and worsening of skin lesions, which negatively impact patient's QoL, were noted in three patients. For one patient, the negative effects on patient wound healing led to a dose reduction of cetuximab and subsequent tumor recurrence soon after [98]. This negative impact of cetuximab in RDEB is likely associated with the important roles of the aforementioned downstream signaling cascades in wound healing processes and exemplifies the challenge of treating tumors in the background of EB. Thus, more targeted strategies, aimed at inhibition of tumor-essential pathways downstream of EGFR are already being investigated and are expected to minimize such adverse effects.

2.3.2 JAK1/2 inhibition

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway mediates cellular responses to a variety of cytokines and growth factors, including IL-6 and EGF, downstream of these ligands binding their cognate receptors. These responses include proliferation, differentiation, migration, survival, and apoptosis, and are dependent on cell- and tissue-type, as well as the context of the signal [101]. In this respect JAK/STAT plays important roles in developmental and homeostatic processes, and is also aberrantly active in numerous cancers, including HN-SCC [102]. Increased levels of phosphorylated STAT3, a downstream effector of JAK, were observed in RDEB-SCC cells over normal keratinocytes, providing rationale for evaluating the effect of the JAK1/2 inhibitor ruxolitinib in a murine xenograft model of human RDEB-SCC [103]. In this preclinical study, ruxolitinib effectively reduced tumor mass, when administered either orally or topically onto tumors, because reduced STAT3 signaling led to decreased cell proliferation. These observations argue that ruxolitinib may be a promising anticancer drug for RDEB-SCC. When ruxolitinib was used with the aim to counteract the fibrotic processes in the skin of type VII collagen hypomorphic mice, a reduction of phosphorylated STAT3 in fibroblasts and SCC *in vitro*, but only limited therapeutic benefit against fibrosis-driven mitten deformities in type VII collagen hypomorphic mice was observed [104]. Additionally, the drug was not well tolerated by the mice and, even more importantly, treatment delayed wound healing, highlighting that caution and rigorous evaluation is warranted before its clinical application in patients.

2.3.3 Polo-like kinase 1 inhibition

Due to the aggressive and metastasising nature of RDEB-SCC, which is atypical of UV-induced cutaneous SCCs that arise in the general population, gene expression assays were performed to identify differentially regulated genes that might account for this difference in tumor behavior. Among the handful of genes identified was polo-like kinase 1 (PLK1) [105], a serine/threonine protein kinase which was overexpressed in a number of different tumors [106]. Blocking PLK1 leads to mitotic arrest, inhibition of cell proliferation and apoptosis. Notably, cells were more sensitive to PLK1 inhibition when p53 was defective [107]. *TP53* is frequently mutated in RDEB-SCC [92] highlighting PLK1-inhibition as a potential strategy to selectively

target tumor cells over normal cells which exhibit normal p53 function. Several small molecules are under investigation for their ability to target PLK1 signaling in cancer, and results from these studies can be leveraged to facilitate the evaluation of these agents also for clinical use in RDEB patients. A study by Atansova et al., identified rigosertib (or ON-01910) among six different small-molecule inhibitors of PLK1 as a strong and selective inducer of apoptosis in RDEB-SCC cells. Its pre-clinical evaluation in a murine xenograft model demonstrated inhibition of tumor growth without obvious toxicity, laying the path for a multi-center phase II clinical trial in RDEB patients with late stage, metastatic, and/or unresectable SCC. [64] (NCT03786237).

2.3.4 Immune checkpoint blocking

Immune checkpoints are molecules that are either able to turn on (co-stimulatory molecules) or turn off (inhibitory molecules) immune signaling, generally referring to the activation of responses in T cells. Tumors have developed mechanisms to exploit these immune checkpoint molecules in order to evade immune surveillance and escape clearance by *e.g*. cytotoxic T cells [108]. Such immune checkpoint molecules include CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and PD-1 (programmed death-1). The latter is predominantly expressed on T cells, and by binding to its ligands PD-L1 and PD-L2 expressed on tumor cells, induces a negative signal that leads to effector T cell suppression [109]. Blocking these interactions using specific antibodies leads to reactivation of the immune system and improvement of anti-tumor immune responses. Remarkable anti-tumor effects were achieved with an antibody targeting CTLA-4 (ipilimumab), increasing median overall survival in metastatic melanoma patients [109]. Even better outcomes in survival rate have been observed with pembrolizumab, an anti-PD1 receptor antibody [110]. Furthermore, beneficial evidence in clinical trials using anti-PD1 treatment in advanced HN-SCC [111], and also locally advanced/metastatic SCC [112] support PD-1 blocking as well in RDEB SCC. In this respect, 2 reports can be found in the literature describing the use of anti-PD1 blocking antibodies in RDEB-SCC patients. The first case described the use of pembrolizumab (PD-1 antibody) as second line therapy after cetuximab treatment. Pembrolizumab was partly combined with other therapeutic approaches including intralesional administration of talimogene laherparepvec (T-Vec; oncolytic virus) into metastatic tumors, radiotherapy and anti-EGFR monoclonal antibody (panitumumab). Wound healing was not impaired during the late stage of the disease. The patient died due to tumor progression 18 months after starting pembrolizumab treatment [100]. Most recently, Khaddour et al. report on an RDEB patient with metastatic SCC who was treated with cemiplimab (monoclonal anti-PD1) every 3 weeks in combination with radiotherapy. During the 14-month follow-up the patient showed a durable response with no signs of immune-related adverse events [113]. These observations support conducting controlled clinical trials to assess the efficacy of PD-1 blockade in this patient group. Noteworthy, another anti-PD1 antibody (nivolumab) is currently under evaluation in a multi-centre phase II clinical trial for the palliative treatment of EB patients with advanced or metastatic squamous cell carcinomas (EudraCT: 2016–002811-16) [6].

3. Conclusions

In the last decade, the number of clinical trials registered for the evaluation of therapies against various primary and secondary pathologies associated with the various forms of EB has risen dramatically (>70 clinical trials; clinicaltrials.

gov). They reflect the progress in the optimization of previous gene therapeutic approaches, discovery and advancement of novel gene editing technologies, and the increase in our understanding of the molecular and cellular mechanisms underpinning the nature of these EB-related complications. Drug repositioning has largely been prioritized, as leveraging the existing pharmacological and safety data represents the fastest and most economical route to clinical trials, and when successful, to marketing approval for EB. To further support the development of new therapy options for rare diseases like EB, several programs, like the orphan designation program by the European Medicines Agency (EMA) have been launched.

This is good news for patients, but also creates challenges for the recruitment of sufficient participants to the various trials, due to the rarity of the disease, strict inclusion criteria, and the disinclination of patients to participate. This heightens the risk of recruitment failure and the inability to meet statistical endpoints, resulting in extended trials that come at increased costs. Surmounting these challenges will require close collaboration within the entire EB community, to establish an international patient registry, incentivize patient participation, address logistical and regulatory aspects of multi-center trials, and allow for new outcome measures and the development of statistical methods for small cohorts. In parallel, applying current state-of-the-art methods that maximize acquisition of multi-modal data from patient samples, alongside the continuing advances in artificial intelligence, will further support the development of new therapies at various levels, starting from *in silico* drug discovery to establishing new means for measuring patient-relevant trial outcomes.

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Conflict of interest

The authors declare no conflict of interest.

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