Review

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Novel and emerging therapies in the treatment of recessive dystrophic epidermolysis bullosa

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Summary **Epidermolysis bullosa (EB) is a clinically and genetically heterogeneous group of inherited blistering diseases that affects ~ 500,000 people worldwide. Clinically, individuals with EB have fragile skin and are susceptible to blistering following minimal trauma, with mucous membrane and other organ involvement in some subtypes. Within the spectrum of EB, ~ 5% of affected individuals have the clinically more severe recessive dystrophic (RDEB) variant with a prevalence of 8 per one million of the population. RDEB is caused by loss-of-function mutations in the type VII collagen gene, *COL7A1*, which leads to reduced or absent type VII collagen (C7) and a paucity of structurally effective anchoring fibrils at the dermal-epidermal junction (DEJ). Currently, there is no cure for RDEB, although considerable progress has been made in testing novel treatments including gene therapy (lentiviral and gamma retroviral vectors for *COL7A1* supplementation in keratinocytes and fibroblasts), as well as cell therapy (use of allogeneic fibroblasts, mesenchymal stromal cells (MSCs), and bone marrow transplantation (BMT)). Here, we review current treatment modalities available as well as novel and emerging therapies in the treatment of RDEB. Clinical trials of new translational therapies in RDEB offer hope for improved clinical management of patients as well as generating broader lessons for regenerative medicine that could be applicable to other inherited or acquired abnormalities of wound healing or scarring.**

Keywords: Epidermolysis bullosa, treatment, protein, cell, gene

1. Introduction

Epidermolysis bullosa (EB) comprises a phenotypically diverse group of inherited blistering diseases that affect the skin and, in some subtypes, mucous membranes and other organs (*1*). Clinically, individuals with EB have fragile skin and are susceptible to blistering following minimal trauma. Depending on the level of blistering within the dermal-epidermal basement membrane zone, EB is classified into four main categories; simplex, junctional, dystrophic and Kindler syndrome (*1*). The sub-classification of EB extends to over 30 clinical subtypes with pathogenic mutations in at least 18 distinct genes (*2*). Within the spectrum of EB, ~ 5% of affected individuals have the clinically more severe recessive dystrophic (RDEB) variant. Dystrophic EB is caused by mutations in the *COL7A1* gene encoding type VII collagen (C7) the major component of anchoring fibril adhesion structures that link the epidermal basement membrane to the subjacent dermis (*3,4*). Inheritance of DEB can be autosomal dominant (DDEB) or autosomal recessive (RDEB) and all cases result from *COL7A1* mutations; more than 1,500 mutations have been reported globally, most of which are specific to individual families (*5*). In RDEB, the *COL7A1* pathology usually involves bi-allelic loss-of-function mutations with point mutations or small insertions/deletions leading to nonsense, splice site, frameshift, or occasionally missense mutations disrupting C7 synthesis, secretion and polymerisation and thereby causing structurally defective anchoring fibrils leading to skin fragility. The most severe forms of RDEB are associated with a complete absence of expression of C7 in skin basement membrane leading to no discernible anchoring fibrils (*6*). In this review, we

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asses novel and emerging therapies in the treatment of RDEB.

2. Current management: Symptoms and complications

The management of RDEB remains complex with no curative therapy currently available. The main principle of care is to manage blisters and erosions, control infection and prevent complications. Symptom relief is very important as both pain and itch have severely deleterious impacts on quality of life. In RDEB, blisters form following minor trauma and/or friction. These blisters need to be lanced to prevent extension of the blister and further skin damage. Pain is a common and constant feature seen in patients with RDEB and arises from four major sources: skin, pain associated with procedures, bone and gastrointestinal (*7*). For skin care, semi-occlusive dressings that are non-adhesive such as silicone and foam dressings are preferable for treating erosions and reducing skin pain as they absorb exudate and offer some physical protection, thereby providing a moist, clean barrier against bacteria (*8*). Opioids in the form of morphine, oxycodone, codeine and fentanyl given by a variety of routes including oral, subcutaneous and sublingual are an effective method of relieving most types of pain in RDEB (*9*). For oesophageal pain, H2 blockers and proton pump inhibitors for gastro-oesophageal reflux can be used and systemic steroids can be utilised during episodes of acute oesophageal blistering (*10*). Tricyclic antidepressants such as amitriptyline and doxepin taken orally have anecdotally been shown to be beneficial to manage pain in junctional EB (*11*). Pruritus is a common problem and often correlates with the severity of EB, with RDEB individuals often experiencing significant skin itching (*12*). The primary cause of pruritus in RDEB remains unclear but has been postulated that wound healing and inflammation may contribute to an itch-scratch-blister cycle leading to further skin damage (*13*). Menthol containing, oil-based products may be partially helpful in relieving itch (see *www.debra-international.org* for best practice guidelines).

Oral care is difficult in RDEB due to microstomia, ankyloglossia and vestibule obliteration and so there is a tendency to develop dental abscesses and periodontal disease, both of which can cause pain (*14*). Caries in RDEB can be reduced through regular dental follow up to optimise oral hygiene and professional cleaning with fluoride therapy (*15*). Extraction of teeth was previously considered the mainstay of treatment (*16*) but today prevention of dental disease is the main aim with dentists working closely as part of a multidisciplinary approach (*17*). Oral pain can be minimised by rinsing the mouth with coating products such as sucralfate or with the use of topical anaesthetics (*18*).

Insensible losses and thermal dysregulation from chronic wounds leads to a hypercatabolic inflammatory state requiring an increased calorie intake (*19*). As a result, the severity of EB often correlates with malnutrition and so RDEB patients often have an inadequate nutrition with growth retardation commonly seen in at least half of all children with RDEB (*19*). One consequence of inadequate nutrition is pubertal delay and short stature. In most patients with RDEB, bone mineral density is reduced due to poor nutritional status, low 25-[OH] vitamin D levels and reduced mobility (*20*). In RDEB, bone mineral density and serum bone profile should be monitored and managed with the use of calcium and vitamin D supplements and bisphosphonates to reduce the risk of fractures (*21*). If pubertal delay is present in RDEB, it is important to attain age appropriate secondary sexual characteristics for psychological reasons and to optimise growth and acquiring peak bone mineral content, therefore, hormonal induction of puberty is often recommended (*22*).

3. Infection control

Extensive areas of denuded skin pose a risk of skin infection due to the accumulation of serum and moisture that enhances the accumulation of bacteria. Prevention and management of infection is important, as wounds that are chronically colonised heal poorly and slowly (*23*). In critically colonised wounds, the bacterial load can be reduced with topical agents such as diluted bleach baths, topical antiseptics and topical antibiotics (*24*). Wounds showing clinical evidence of frank infection require administration of systemic antibiotics with the choice based on culture and sensitivity results.

4. Surgery for contractures

Blisters and wounds in RDEB heal with scarring. This scarring leads to contractures and is most notable on the hands and feet (*25*). The changes affecting the hands include flexion contractures of the interphalyngeal joints, metacarpophalyngeal, and wrist joints. In severe forms of RDEB a "mitten" deformity develops with epidermal "cocooning" that encases the hand (*26*). With minor trauma to the hands and feet, ulceration occurs which can be followed by fibrinous adhesions and scarring, destroying the web spaces and progressing to the finger tips leading to pseudosyndactyly. The term pseudosyndactyly is used as the dermis of the adjacent fused digits remains and separates the fused digits. Pseudosyndactyly of the hands and feet starts in childhood and is characteristic of severe forms of RDEB (*27*). The formation of scar tissue and contractures causes pain when extending the affected joints (*7,9*). As dermis abuts dermis in the fused digits, surgery releasing the contractures can exploit this level of fusion, although finding a distinct plane of tissue separation can be difficult in older children and adults. Despite the complexity of surgery, intervention is often

successful in releasing the contractures and separating the fingers, although recurrence of pseudosyndactyly typically occurs. Skin grafting is often required and post-surgical splinting to minimise the speed of recurrence is challenging (*26,28*).

5. Squamous cell carcinoma

The most serious complication associated with RDEB is the development of clinically aggressive squamous cell carcinoma (SCC) often arising in areas of non-healing cutaneous wounds (*29*). Based on a US nationwide registry of EB patients, the cumulative risk of first SCC development in severe generalised EB is 7.5% by the age of 20 years. This risk increases to 67.8% by the age of 35, 80.2% aged 45 and 90.1% by the age of 55 (*29*). Approximately 80% of RDEB patients that develop SCC generally die of metastatic disease within 5 years of excision of the primary lesion (*29*). SCCs in RDEB can be multifocal and multiclonal with multiple primary tumours co-existing in one individual (*30*).

Following a systematic literature review and expert consensus, recommendations have been made on the management of cutaneous SCC in EB (*31*). Wide local excision is considered the treatment of choice for EB-associated SCCs. Imaging with a PET-CT scan evaluates distant disease and should underlying vessels, nerves or tendons be involved, then more radical surgery such as amputation may be more appropriate (*31*). Under circumstances when there has been local recurrence of disease or regional or distant metastasis, non-surgical treatment such as radiotherapy or chemotherapy may be considered. Topical preparations such as photodynamic therapy and 5-fluorouracil have been used in a small number of patients with in-situ disease (*31*). When using radiotherapy, consideration needs to be given to severe desquamation that can follow larger total radiation doses. Conventional chemotherapy has been used in cases of advanced EB SCCs (*32-35*). Agents have included cisplatin, carboplatin, paclitaxel, fluorouracil, doxorubicin and methotrexate. Partial remission has been described in some reports although follow up data are limited. Newer biologic agents such as epidermal growth factor receptor (EGFR) antagonists and tyrosine kinase inhibitors have been used in non-EB SCCs (*36-38*), but reports of their use in EB are few. Cetuximab, a monoclonal antibody that binds the extracellular domain of EGFR has shown favourable results in metastatic EB SCCs strongly expressing EGFR, although numbers of cases are limited and long term survival remains poor (*39,40*). Systemic retinoids have been trialled in RDEB as a chemopreventative agent to reduce the risk of SCC. A phase 1 trial of isotretinoin in twenty RDEB patients (*41*) showed no adverse reactions at a low dose of isotretinoin however, increased mechanical fragility was observed at therapeutic doses and so currently, retinoids are not recommended for long term chemoprophylaxis.

6. Wound grafting and topical therapies

A number of biological dressings and wound grafting approaches have been used to treat intractable ulcers in RDEB (*42-46*). Autologous and allogeneic skin grafting have been developed for RDEB with some reported success, mostly in small case series or anecdotal reports. In one study, cultured epidermal autograft (CEA) was manufactured by taking a full-thickness biopsy specimen of skin from an RDEB subject and culturing keratinocytes to confluence. The resultant CEA was then grafted onto a designated area of ulceration with epithelialisation observed 2 weeks later (*42*).

Allogeneic cultured dermal substitutes (CDS) have also been used to treat intractable ulcerated wounds in patients with RDEB (*44,47*). Apligraf® (Organogenesis, Canton, MA, USA) is an allogeneic cultured skin substitute consisting of keratinocytes and fibroblasts supported on a scaffold and was initially used in the treatment of venous ulcers. However, Apligraf® has also been used to treat EB skin ulcers with benefit, although mainly in subtypes of EB other than RDEB (*43,44*).

CDS have been used in several patients with RDEB with reported success (*45,46*) although long term improvements may be limited and repeated preparation and application of skin grafts may not be practicable or economically feasible.

Alternatively, amniotic membrane, which possesses biological properties that can promote wound healing (*48*), has been used in EB to promote healing of chronic wounds (*49*). In a retrospective, proof-of-concept study, amniotic membrane grafting was efficacious in promoting the healing of non-healing wounds in EB with a reduction in pain but complete re-epithelialisation was not achieved (*49*). An additional study in DEB examined clinical application of amniotic membranes if the wound was debrided and found there was spontaneous reepithelialisation in a week and pain and immobility improved within hours (*50*).

Placental material has also been used to manage acute and chronic wounds. Cryopreserved placental membrane (CPM) (Grafix, Osiris Therapeutics, Inc., Columbia, Md.) is a cellular matrix composed of placental membrane matrix that provides the wound with mesenchymal stem cells, neonatal fibroblasts, epithelial cells, growth factors (GFs), and angiogenic factors and has been licensed for the management of EB (*51,52*). Although trial data for RDEB are lacking, CPM showed superior results to standard wound care in a randomised controlled trial comparing the two treatment modalities to treat diabetic foot ulcers, and finding that wound closure at 12 weeks was significantly higher in the CPM group (62% in the CPM arm vs 21% when standard wound care was used) (*53*).

Acellular dressings with collagen derived from a variety of sources have also been utilised to improve wound healing in RDEB (*54*). The rationale for their use,

includes observations that type I collagen may decrease MMP activity and act as an anti-inflammatory agent by binding pro-inflammatory cytokines (*55*). Integra® (Integra LifeSciences, Plainsboro, NJ) is a bilayer wound dressing with acellular bovine collagen and chondroitin-6-sulphate. Helicoll® (Encoll, Fremont, CA) is a single-layer acellular matrix of purified bovine type 1 collagen and has been trialled in patients with RDEB with the primary outcome being wound size measurement (*56*), with a statistically significant improvement in wounds treated with Helicoll® compared to standard dressings. However, upon discontinuation of the type 1 collagen treatment, wounds that had re-epithelialised, soon broke down again with recurrent ulceration.

In addition to wound grafting, topical therapies are also being developed to aid wound healing in RDEB such as thymosin β4, a small molecular weight protein involved in cell proliferation, migration and differentiation, as well as actin polymerisation, which appears to enhance epithelial wound healing when applied topically to wounds in animal studies. The basis of the positive response may involve promoting the migration and adherence of keratinocytes on wounds, and the upregulation of one or more extracellular matrix proteins, particularly laminin-332. A clinical trial to explore the potential of thymosin β4 to promote wound re-epithelialisation in EB was initiated in 2005; this was a randomised double-blind study involving three concentrations of the agent and a placebo control. However, the study had to be terminated early due to lack of subject recruitment, although no adverse events were reported in those who participated (*57*).

Topical growth factors have been used in wound healing in venous leg ulcers (*58*) and diabetic foot ulcers (*59*). However, a topical preparation of PDGF (platelet-derived growth factor) named Regranex® (Smith and Nephew, London, UK) was trialled in a randomised, placebo controlled, double blind trial which showed no significant improvement in the healing of diabetic foot ulcers (*59*). Generally, however, the overall efficacy of topical growth factor preparations has been relatively disappointing, and there have been no reported studies in RDEB.

7. Systemic treatment

Before the genetic basis of dystrophic EB was discovered, ultrastructural studies indicated possible collagen degradation and phagocytosis of collagen fibrils in areas of blistering in RDEB skin (*60*). Thus early attempts at systemic treatment for RDEB focused on inhibiting collagenase. Phenytoin, an anticonvulsant that also has properties as a collagenase activity inhibitor, was trialled in 17 unselected RDEB patients (*61*). After up to a maximum of 15 months of therapy, blisters and erosions were significantly decreased in most of the patients (*61*). In 1992, however, a multi-centre randomised, placebo-controlled, double blind, cross over study of phenytoin in RDEB was performed which showed unequivocally that phenytoin had no significant therapeutic effect (*62*). Thus, there is currently absolutely no clinical rationale for the ongoing prescribing of phenytoin for the treatment of RDEB.

Following on from the proven failure of phenytoin therapy, but still pursuing the anti-collagenase strategy, minocycline was trialled in two patients with DEB (*63*), on the basis that tetracyclines (including minocycline) have anti-collagenase activity (*64*). After commencing minocycline at a dose of either 100mg twice daily or 50mg three times daily blistering was reduced in both subjects (*63*). Similar benefits have also been reported in a patient with dominant DEB. Regarding mechanism of action, it has been shown that levels of matrix metalloproteinase-9 (MMP-9) are raised in RDEB blisters (*65*) and it was thought that the clinical improvement might be due to inhibition of MMP-9 by minocycline (*66*). Nevertheless, minocycline also has a tendency to induce skin hyperpigmentation as a side effect. To date there has been no larger clinical trials to assess clinical use of minocycline in RDEB and thus its use cannot be recommended for routine treatment.

Other antibiotics have been trialled in RDEB, including trimethoprim for its anti-inflammatory effects based on diminished chemotaxis of polymorphonuclear leukocytes, modification of complement pathways and inhibition of MMPs (*67*). In a proof-of concept double blind randomised cross-over trial comparing trimethoprim to placebo in RDEB, there was a trend towards improved wound healing with trimethoprim compared to placebo (*68*) although further assessment will be required before trimethoprim might be recommended for routine clinical use. Another preparation that is able to regulate MMP activity *in vitro* and *ex vivo* is the green tea extract, epigallocatechin-3-gallate (EGCG) (*69*). A multicentre, randomised, crossover, double blind, placebo controlled clinical trial in 17 RDEB individuals evaluated whether a 4 month course of oral EGCG might be efficacious in improving skin impairment (*70*). Despite the EGCG group having less daily blisters and shorter wound healing times, however, the study failed to demonstrate statistical significance between the two groups. Thus no formal recommendations can be based about the use of oral EGCG in RDEB based on this single study.

Regarding other anti-inflammatory drugs, ciclosporin was discovered to have clinical benefits in the treatment of DEB when prescribed to prevent graft rejection in a child with DEB (*71*). However, given the increased risk of skin malignancy in RDEB, long term use of ciclosporin cannot be recommended. For other immunosuppressant drugs, a randomised controlled double blinded study in 35 patients with DEB was conducted to evaluate ciclosporin versus mycophenolate mofetil (MMF). The percentage of improvement in the

ciclosporin group was statistically significantly higher than the MMF group but there was no difference in the number of new blisters or the rate of healing of new blisters between the groups (*72*). As for ciclosporin, however, long term use of MMF in RDEB is not advisable.

In other anecdotal reports, the tumour necrosis factor alpha (TNF-α) inhibitor etanercept has been assessed in RDEB (*73*). Etanercept is a fusion protein produced by recombinant DNA and is used to treat a variety of disorders mediated by excess TNF-α such as psoriasis and psoriatic arthritis. A 29-year-old woman with concomitant DEB and psoriatic arthritis was given etanercept to treat her psoriatic arthritis. A progressive improvement in her DEB was noted in the first 3 months of treatment with subcutaneous etanercept, 50mg twice a week, with an improvement in pruritus and fewer blisters; notably, the clinical improvement persisted over the 3 years she was receiving etanercept (*73*).

A patient with RDEB undergoing bone-marrow transplantation (BMT) for her disease (see bone marrow transplantation section) observed that there was a significant improvement in her wound healing during autologous peripheral blood stem cell mobilisation with systemic granulocyte colony-stimulating factor (G-CSF) prior to the transplant (*74*). Based on this anecdotal finding, a pilot trial was designed to confirm the safety of daily doses of G-CSF, (10 μg/kg/dose) in 6 RDEB and one DDEB subject. The patients were re-evaluated at Day 7 and for all patients combined, median reductions of 75.5% in wound size and 36.6% in blister/erosion counts were observed. G-CSF was well tolerated and no adverse events were noted. At the request of some individuals, further injections of G-CSF were administered which demonstrated that the response was reproducible and safe (*74*).

In addition to strategies employed to correct the causative pathology in RDEB, there is also a need to treat collateral pathology such as scarring. The functional limitation of movement secondary to extensive scarring and fibrosis is a major complication of RDEB. A hypomorphic mouse model suggests that this scarring and fibrosis is driven by transforming growth factor beta-1 (TGF-β) signalling, as reflected by transition of dermal fibroblasts to myofibroblasts with capacity for ECM production (*75*). Losartan, an angiotensin II type 1 receptor antagonist, that is primarily used to treat hypertension, has also been shown to possess anti-fibrotic effects resulting from suppression of TGF-β1 *via* angiotensin II type 1 receptor mediated down regulation of TGF-β1 activators such as thrombospondin 1 (TSP-1). TGF-β activity is elevated in injured RDEB skin, and so by targeting TGF-β activity, fibrosis may be reduced and in turn, delay mitten deformity development (*75*). In murine studies, losartan has been shown to reduce TGF-β levels in RDEB cells *in vitro*, and in the skin and the circulation of RDEB mice. As a result of reduced TGF-β activity, there was significantly slower progression to fibrotic digit fusion and mitten deformities (*76*). The role of TGF-β signalling has been highlighted as a potential modifier of disease severity following the study of monozygotic twins with RDEB with markedly different clinical phenotypes and similar amounts of C7 expression (*77*). In this study, genome wide expression analysis in twins' fibroblasts showed differential expression of the genes associated with TGF-β pathway inhibition. Decorin, a skin matrix component with anti-fibrotic properties was more expressed in the skin of the less severely affected twin. Fibroblasts from the more affected twin were characterised by enhanced α-smooth muscle actin and plasminogen activator inhibitor 1 expression, collagen I release and collagen lattice contraction.

Preclinical studies are also ongoing to evaluate the reparative potential of high mobility group (HMG) proteins, specifically by mobilising key epithelial progenitors from bone marrow which are then recruited to damaged RDEB skin. Murine studies have demonstrated that one of the HMG proteins, high mobility group box-1 (HMGB-1), is rapidly released from hypoxic keratinocytes, such as from blister roofs, and upon release into the circulation, reparative epithelial progenitor cells (Lin-/PDGFRα+) are mobilised from within the MSC-BM population (*78*). These cells are recruited along a concentration gradient to the area of hypoxic skin damage. Differentiation of these cells into keratinocytes (rather than fusion) was clearly demonstrated, with persistence of the differentiated BM cells in the skin after several renewals of the murine epidermis, data which support engraftment of a murine BM population that has generated keratinocyte stem cells (*78*).

8. Cell therapies

8.1. *Allogeneic fibroblasts*

Fibroblasts have the capacity to synthesise C7 as well as modulating wound healing (*79*). On this basis, a number of RDEB murine and human studies have been conducted injecting allogeneic normal human fibroblasts intradermally with the aim of potentially increasing C7 expression and also improving wound healing (*80*).

A proof-of-concept study in 5 RDEB individuals demonstrated that a single intradermal injection of allogeneic fibroblasts (5×10^6 cells injected into the superficial dermis over ~ 1 cm^2) increased *COL7A1* expression for at least 3 months in most subjects (*80*). The study also demonstrated the low immunogenicity of allogeneic fibroblasts and lack of host response at an immunological and histological level. The injected cells were not detectable at 2 weeks post-injection, the time-point at which an increase in C7 protein at the DEJ was

seen. In murine studies, it has been suggested that this increase in C7 protein at the DEJ may be secondary to donor fibroblasts releasing wild-type full length C7 that can be incorporated into the DEJ for the short time that these donor fibroblasts are present (*81*). Of note, in the human studies, the increase in C7 was most apparent in RDEB individuals who had some baseline expression of C7 compared to those who had a complete absence of the protein. The source of the new C7 is likely to reflect upregulation of the RDEB subjects' own mutant, but partially functional C7, a mechanism supported by a lack of new normal-appearing anchoring fibrils. A further study showed that a single injection of allogeneic fibroblasts could increase *COL7A1* expression for 3-6 months and C7 protein for 9-12 months (*82*). The expression of heparin binding-EGF-like growth factor (HB-EGF) was thought to mediate this increase in endogenous C7 expression (*82*).

With regard to wound healing, a phase II double-blinded, randomised, controlled trial in RDEB patients comparing injections of allogeneic cultured fibroblasts in suspension solution versus suspension solution alone, with the injections given across eroded areas found that in both arms there was a reduction in erosion size, suggesting that perhaps the trauma of either injection might, at least in part, be responsible for the clinical responses (*83*). On the other hand, a further prospective, randomised, double-blind, within-patient, vehicle-controlled trial of subjects with RDEB was conducted in 11 patients. Twenty-six erosions were treated; 14 with a single treatment of 5×10^6 allogeneic fibroblasts per linear cm of erosion margin and 12 with vehicle. Fibroblast injections produced a greater reduction in erosion area than did vehicle alone during the first 28 days. After 28 days, there was no significant difference between fibroblasts and vehicle although further injections were not administered (*84*).

8.2. *Mesenchymal stromal cells*

Multipotent mesenchymal cells are found in several tissues, including the bone marrow (*85,86*) and have the ability to migrate to injured tissue and stimulate tissue regeneration, thus making this therapy potentially relevant to RDEB wounds. The clinical use of MSCs in RDEB was first reported in a 13-year-old and 25-year-old patient from Chile in 2010 (*87*). The MSCs were derived from the bone marrow of healthy, unrelated individuals and injected intradermally. Both subjects had clinically severe blistering with a complete absence of C7 expression. Either 0.5×10^6 MSCs or vehicle were injected into both intact and chronically ulcerated sites. At week 12, wounds treated with MSCs had almost healed compared to sites treated with placebo with benefits lasting for 4 months post injection. Thereafter, skin fragility resembled baseline with ulceration. New C7 was seen in a linear pattern at the junction between the epidermis and dermis, suggesting that intradermal administration of allogeneic MSCs may lead to *de novo* C7 expression in the skin as well as prevention of blistering and improvements in wound healing in patients with RDEB.

Subsequently, El Darouti *et al*. (*88*) conducted a double-blind study, randomising 14 patients with clinically severe RDEB into two equal groups. Both groups received intravenous MSCs derived from healthy bone marrow aspiration from one healthy parent but group one was also given 5 mg/kg/day of ciclosporin to reduce inflammation or protect against rejection with the patients in group two receiving a placebo suspension. Both groups were seen fortnightly for 12 weeks and were reported to have fewer new blisters, to have an increased rate of wound healing, and to demonstrate new anchoring fibrils on skin biopsies. Two individuals demonstrated clinical benefit at 12 months, whereas the improvements in the remainder peaked 3 months after infusion and waned thereafter.

Petrof *et al*. (*89*) enrolled 10 children aged 1-11 years in the U.K. with RDEB who had partial or complete absence of C7 protein, in an open-label, phase I/II clinical trial. Each child received three IV infusions of either 20×10^6 cells per infusion (weight $\leq$ 20 kg) or 40×10^6 cells per infusion (weight > 20 kg) (equivalent to $1\text{-}3 \times 10^6$ cells per kg) of BM-MSCs on days 0, 7 and 28. No severe adverse events occurred (other than the transient noxious smell associated with the preservative dimethyl sulphoxide). Skin biopsies revealed no increase in C7 and no new anchoring fibrils at day 60 post infusion. One subject showed no clinical benefit, whereas two had sustained improvement at one year, and in the others there were transient improvements such as less skin redness, less skin pain and itching, and better wound healing that lasted for 4-6 months after the third infusion of MSCs. The optimal dosing, route of administration and consequences of multiple repeat dosing of allogeneic MSCs in RDEB has yet to be fully evaluated. However, murine studies have shown the impact and superiority of high density intradermal injections of MSCs compared to fibroblasts, suggesting that further human clinical trials are needed if the maximal benefits of MSC cell therapy in RDEB are to be realised (*90*).

The mechanism by which MSCs lead to a clinical improvement in wound healing in RDEB has not yet been established but seems to be indirect and trophic through the release of various growth factors and cytokines (*91*), *i.e*. without the need for the MSCs to engraft. MSCs express tumour necrosis factor alpha (TNFα)-stimulated protein 6 (TSG-6), which in other studies has been associated with an improvement in wound healing and downregulation of B-cell proliferation, monocyte maturation, secretion of IFN-γ and TNF-α at wounded tissue sites (*92*), while also promoting increased secretion of anti-inflammatory

IL-10 from macrophages (*93*). In addition to TSG-6, MSCs also mediate immunosuppression through the secretion of nitric oxide, transforming growth factor-beta (TGF-β) and indoleamine 2,3-dioxygenase (*94*).

Regarding other cells, potentially with stem rather than stromal functionality, human umbilical cord blood derived unrestricted somatic stem cells (USSCs) have shown potential to regenerate RDEB skin in animal models (*95*). In murine models, it has been shown that USSCs express C7 and accelerate wound healing when injected intradermally in mice that have full-thickness excisional wounds (*96*). An intradermal injection of USSCs modified with a luciferase reporter gene, injected at a distant site to the wound revealed specific migration to the wound (*96*). These data suggest that CB-derived USSCs may contribute to wound repair and may be worth exploring as cell therapy for patients with RDEB. In terms of optimizing MSCs for clinical use, preconditioning of MSCs with TGF-β, TNF-α, and SDF-1α, induces a simultaneous upregulation in *COL7A1*, *TSG-6*, and *CXCR4* which results in a six to eight-fold increase in *COL7A1* expression by MSCs (*97*). This pre-conditioning increased C7 levels towards the 30% of the amount of wild-type C7 believed to ameliorate the blistering seen in RDEB (*75*). Such pre-conditioning effects, however, have yet to be assessed therapeutically in humans.

8.3. *Bone marrow transplantation*

Following the effectiveness of bone marrow (BM) stem cells in murine RDEB (*98,99*), a clinical trial of whole bone marrow transplantation (BMT) was performed in children with RDEB.

In 2010, Wagner *et al*. (*100*) reported use of high dose chemotherapy to immunoablate individuals with RDEB to permit more reliable lymphohaematopoietic engraftment, followed by unfiltered whole bone marrow transplantation, usually from a tissue-matched sibling donor. Seven patients entered the trial and 6 underwent BMT. One patient died before the BMT because of heart failure, possibly related to cyclophosphamide toxicity and pre-existing renal failure. All RDEB subjects had more than 50% body surface area coverage with blisters and erosions. Following BMT, 3 subjects showed clinical improvement with only 10% BSA involvement and 3 showed an improvement with 25% BSA involvement. A further patient died 6 months post-transplant from infection secondary to graft failure. Of note, donor cells homed to injured skin with increased C7 expression seen at the DEJ in 5 of the 6 subjects. The subject that did not show evidence of increased C7 expression post-BMT was still reported to show an improvement in their clinical status, similar to that seen in the other 5 subjects that did show an increase in C7 expression. Clinical response seems to have been sustained; none of the treated subjects has been cured of their RDEB but several have had markedly fewer blisters in follow up to 8 years post-BMT. Donor-skin chimerism was seen in the skin of BMT recipients (*101*). A substantial number of cells of donor origin were found in BMT recipient skin, confirming that donor cells home to injured skin in patients with severe RDEB. Donor cells of both haematopoietic (CD45+), and non-haematopoietic, non-endothelial cells (CD45-, CD31-) were found in the epidermis and dermis of BMT recipients, although donor non-haematopoietic cells were considered to be the most likely source of new C7 (*101*). Despite the increase in C7 expression, there was a lack of mature anchoring fibrils on transmission electron microscopy (TEM), although later evaluation will be needed given the several years anchoring fibril maturation may take.

Regarding the interconnectivity between BM cells and skin repair, the release of HMGB-1 from hypoxic keratinocytes and the mobilisation of Lin-/PDGFRα+ epithelial progenitor cells from bone marrow to the circulation and differentiating into keratinocytes capable of generating new C7 in the skin, supports the potential mechanism of action of BMT (*78*). However, the homing of these cells to injured skin post-BMT has not yet been fully established. Reports suggest that the C-X-C type chemokine ligand 12 (CXCL12), known as stromal cell-derived factor 1α (SDF-1α), and its receptor, CXCR4 may direct the migration of progenitor cells to various tissues (*102*). The transcription factor hypoxia inducible factor-1 alpha, HIF-1α, in endothelial cells in ischaemic tissue regulates the expression of SDF-1α, enabling CXCR4+ progenitor cells to home from the circulation to target ischaemic tissue (*103*). Overall, despite the clinical data, the precise mechanism by which BMT leads to clinical improvement has not yet been fully elucidated. Of clinical significance, however, immunoablative conditioning in RDEB pre-BMT has been associated with mortality rates in excess of 25%. To lessen mortality, several refined stem cell transplantation protocols have been developed that focus on reduced intensity conditioning (RIC). Combination conditioning has been reduced from using busulfan, fludarabine, and cyclophosphamide to combination therapy with fludarabine and low doses of cyclophosphamide and radiation (*101*), although further refinements continue to be applied. Thus far, it appears that RIC is associated with less toxicity and relatively good disease amelioration, but published data are currently lacking.

8.4. *Grafting revertant mosaicism skin/keratinocytes*

In patients with various inherited cutaneous diseases, patches of spontaneously appearing normal skin can be seen where the inherited mutation has genetically corrected itself in those sites. This phenomenon is referred to as revertant mosaicism or "natural gene therapy" (*104*) and a key goal has been to try to exploit these natural events in the treatment of

RDEB. Thus far, revertant mosaicism has not been explored therapeutically in RDEB although in some forms of junctional EB, grafting of cultured revertant keratinocytes (*105*) or punch grafting of revertant skin has been undertaken, with sustained improvement in recipient mutant skin sites being demonstrated for the latter (*106*).

The opportunity to expand keratinocytes derived from a patch of revertant mosaicism offers a personalised and patient specific form of therapy. As these cells have naturally corrected part of the deleterious mutation, there is no need for further genetic manipulation. Gostynski *et al*. (*105*) isolated revertant keratinocytes from an individual with generalised intermediate junctional EB and expanded these into epidermal sheets to graft on to areas of mutant skin lacking an epidermis. The surgical approach led to successful grafting although functional benefits were not apparent. Of note, despite cultured keratinocytes displaying 30% reversion, when grafted, less than 3% of keratinocytes remained reverted in the graft; the reasons for this relative loss of reverted cells is not known. More successful was punch graft transplantation of revertant skin in an individual with junctional EB that resulted in successful transfer of the donor cell genotype and phenotype with enhanced expression of laminin-332 and better skin integrity maintained for at least 18 months (*106*). Nevertheless a key challenge is to find methods for higher *in vitro* expansion of revertant keratinocytes as well as being able to more readily identify the revertant skin patches (*107*). One new approach has been to generate inducible pluripotent stem cells (iPSCs) from revertant keratinocytes (see gene therapy section below) (*108,109*), which potentially then offers copious functional cells that can be differentiated into multiple tissue lineages.

8.5. *Gene therapy*

Gene therapy strategies in RDEB aim to provide therapeutic benefit through manipulation of DNA or RNA. Typically, viral mediated *ex vivo* gene transfer approaches have been used whereby the patient's skin cells are cultured, transduced with a viral vector encoding the transgene expressing the wild-type protein and then these gene modified cells can then either be transplanted back *via* grafting of epithelial sheets or skin equivalents (epidermis/dermis), or by intradermal injections (*e.g*. of genetically supplemented fibroblasts). Viral mediated gene transfer has been the preferred gene delivery method, firstly, due to the ability to deliver a transgene and integrate it into the host genomic DNA, and secondly because viral vector approaches achieve higher transduction efficiencies for longer-term gene expression. Gamma retroviral (RV) and lentiviral (LV) vectors have been the main delivery methods for RDEB gene therapy studies, despite the large size of the *COL7A1* cDNA (> 9 kb) (*110-113*). Regarding specific pre-clinical work for RDEB, one study used an LV-mediated system to make intradermal injections of corrected patient-derived RDEB fibroblasts to restore C7 at the dermal-epidermal junction for 4 months in an RDEB skin model (*111*). Moreover, it was subsequently shown that direct intradermal injections of an LV vector containing *COL7A*1 cDNA could produce stable expression of human C7 in fibroblasts and endothelial cells for at least 3 months in a murine model (*114*). To compensate for the large size of *COL7A1*, an RV vector with a truncated *COL7A1* "mini-gene" was first assessed (*115*). Immortalised RDEB keratinocytes could be transduced to express a mini-C7 protein product that improved cell motility, adhesion, and proliferation, although mini-gene therapy approaches have not been pursued to clinical trials.

The first clinical study of *ex vivo* gene therapy for EB was in an individual with junctional EB, with restoration of laminin-332 expression following RV-mediated transfection of epidermal stem cells with the *LAMB3* gene, leading to phenotypic correction in the grafted skin (*116*). Of note, follow up for more than 8 years has shown sustained synthesis of laminin-332 protein with no evidence of blistering, inflammation, tumourigenesis or immune response in the grafted area (*117*). In a second case, the same RV gene therapy protocol was used in an Austrian junctional EB patient in whom *ex vivo* skin gene therapy targeting autologous epidermal stem cells was used to produce five skin sheets each measuring 5 × 7 cm that were grafted onto wounded areas on the patient's thighs; clinical responses in this patient are still being evaluated (*118*).

The first gene therapy trial in RDEB involved grafting of *ex vivo* autologous *COL7A1* gene supplemented epidermal sheets in 4 adults in a phase I clinical trial. In this study, autologous keratinocytes were transduced with GMP grade gamma-RV containing full-length *COL7A1*. Autologous epidermal sheets measuring ~35cm^2 (approximately the size of a playing card) were grafted onto 6 wounds in each of the patients. No serious adverse events were reported and there was C7 expression at the dermal-epidermal junction on graft sites in 90% of biopsies at 3 months, 66% of biopsies at 6 months and 42% at 12 months. Wound healing was variable and generally waned over one year. Longer term follow-up will be required to ascertain long-term efficacy and safety (*119*).

The risk of insertional mutagenesis arising from use of certain classical viral vectors has led to a new generation of self-inactivating (SIN) viral vectors which incorporate deletion of the U3 region of the 3'-long terminal repeat that renders them unable to activate cellular genes in the host's genome. A SIN-LV-based vector was used to deliver full-length *COL7A1* cDNA sequence into patient-derived RDEB keratinocytes and fibroblasts (*110*). This approach gave close to 95% transduction efficiency and demonstrated persistent synthesis and secretion of normal C7 over a 5 month

observation period *in vitro* (*110*). These corrected cells were also able to produce normal anchoring fibrils when grafted onto immunodeficient mice. Other investigators are currently carrying out a clinical trial of SIN-LV vector *COL7A1* addition to autologous fibroblasts for intradermal injection (ClinicalTrials.gov identifier: NCT02493816), and others are developing a SIN-RV vector containing full length *COL7A1* with the aim being to transplant bioengineered skin containing genetically supplemented keratinocytes and fibroblasts (*www.genegraft.eu*).

As an alternative to viral-mediated transduction, a phage-mediated platform has been used to deliver *COL7A1* cDNA into patient-derived RDEB primary epidermal progenitor cells (*120*). The authors used a phiC31 phage integrase, which can integrate large (up to 10 kb) DNA sequences. The experimental data revealed relatively lower transfection efficiency rates (~ 45% at 2 days) compared to viral transduction methods, but through culture expansion and selection of C7-producing cells, a ~ 99% success rate after a 10-day selection period was noted. Moreover, C7 production by epidermal progenitor cells was suggested by persistent expression for 14 weeks, *i.e*. spanning multiple turnover cycles of keratinocytes. The same phiC31 phage integrase platform was subsequently used to correct patient-derived RDEB fibroblasts. Corrected fibroblasts were then injected into an RDEB skin model and were shown to restore C7 expression in the skin (*121*). Nevertheless, the requirement to include the phiC31 integrase gene, the lack of responsiveness to endogenous gene regulation, and the potential for random insertional mutagenesis may be limiting factors for phage therapy.

Cationic polymers such as linear poly (β-amino ester)s (LPAEs) have also emerged as an effective gene delivery vector. Branched poly (β-amino ester) s (HPAEs) have a three-dimensional spatial structure and are thought to improve the interaction of polymers with DNA, prevent DNA degradation by enzymes and increase cellular uptake of polyplexes. HPAEs have not been developed for gene delivery as yet, as synthesising these highly branched polymers remains a technical challenge. A novel design of the HPAEs has been derived from the functional LPAE components to see whether this may provide an effective gene delivery vector. This has been assessed *in vivo* in various cell types including RDEB keratinocytes to deliver therapeutic *COL7A1* cDNA (*122*).

Gene silencing technologies such as RNA interference (RNAi) are useful in dominant forms of DEB, if designed to knockdown the mutant allele without silencing the wild-type allele, with pre-clinical data to support therapeutic use of such an approach (*123,124*). Another methodology, pertinent mainly to RDEB but possibly also dominant disease, is to try to modulate splicing of pre-messenger RNA to induce skipping of the mutated exon. Using 2'-O-methyl antisense oligoribonucleotides (AONs) in an RDEB skin equivalent xenograft model, one or two subcutaneous injections of AONs at doses ranging from 400 μg up to 1 mg was able to induce skipping of exons containing loss-of-function mutations (in exons 73 and 80) and thereby restore C7 expression and anchoring fibril formation (*125*). A further method is to apply spliceosome-mediated RNA trans-splicing (SMaRT) to address target mutations at a post transcriptional level. Splicing is induced in *trans* between the exogenous RNA and target endogenous pre-mRNA *via* an engineered RNA trans-splicing molecule (RTM). Specifically, RV transduction of RDEB keratinocytes with a 3' pre-trans-splicing molecule resulted in correction of full-length C7 expression (*126*). Transduced cells showed normal localisation of C7 at the basement membrane zone in skin equivalents with assembly into anchoring fibril-like structures, *i.e.* demonstrating correction of an RDEB phenotype *in vitro* (*126*). In further work, a 5' RTM capable of replacing COL7A1 exons 1 to 15 in murine keratinocytes was injected into the skin of wild-type mice using a gene gun with vector delivery and expression in the skin (*127*).

Approximately 15% of all pathogenic mutations in *COL7A1* involve premature termination codons (PTCs) that lead to truncated proteins and/or nonsense-mediated mRNA decay (*128*). Both *in vitro* and *in vivo* studies have revealed that aminoglycoside antibiotics can suppress primary PTCs and produce some degree of full length functional protein in genetic disorders such as cystic fibrosis (CF) and Duchenne's muscular dystrophy (DMD) (*129,130*). In RDEB, preclinical analysis has been performed using two RDEB keratinocyte cell lines harbouring nonsense mutations and primary fibroblast cultures from two RDEB patients with nonsense mutations. Aminoglycosides (G418, gentamicin, and paramomycin) were able to induce PTC read-through and restore functional full-length C7. Aminoglycoside therapy may provide a non-invasive option in treating RDEB patients that carry nonsense mutations but has not yet been trialled. Potential toxicity and the extent of the readthrough necessary to generate functional correction, however, remain important considerations that may limit immediate clinical translation.

Genomic editing techniques including zinc-finger nucleases (ZFNs), meganucleases (MN), transcription activator–like effector nucleases (TALENs) (*131,132*) and the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 nuclease system are being developed (*133*), some or all of which may have relevance to RDEB therapeutics.

Moreover, the advent reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) that can differentiate into any cell type, is an exciting new development in RDEB therapy (134). It is possible to correct RDEB fibroblasts through homologous recombination using transcription activator-like effector

nucleases (TALENs) and then reprogram these into iPSCs, which then differentiate into keratinocytes (*135*). Murine studies have also successfully generated iPSCs in culture from multipotent keratinocyte lineages capable of forming a fully developed epidermis (*136*). Subsequently, others have reported successful generation of iPSCs from healthy human skin fibroblasts and individuals with RDEB (*137*). Another study took a different approach using direct injections and teratoma formation which allows spontaneous differentiation of iPS cells into an epidermis (*138*). Regarding new therapeutic opportunities, an approach in which iPSCs generated from naturally corrected revertant RDEB cells could be used to enable the production of autologous epithelial and mesenchymal cells, perhaps paving the way for personalised therapy in EB (*108,109*).

8.6. *Protein therapy*

Given that the essential skin pathology in RDEB is a lack of C7 in epidermal basement membrane, C7 protein replacement therapy has been evaluated using animal models for preclinical studies. Initial studies successfully demonstrated that intradermal injections of recombinant human C7 can lead to incorporation of the new protein specifically into basement membrane of *Col7a1* null mice, resulting in an improvement in the blistering phenotype for up to 2 months (*139*). Furthermore, topical application of human recombinant C7 accelerated wound healing in mice (*140*), and intravenously administered rC7 homed to engrafted RDEB mouse skin and restored C7, anchoring fibrils, and epidermal-dermal adherence (*139,141*).

Concerning larger animal studies, intravenous administration of C7 in a spontaneous animal model of inbred mini Retriever dogs with mild RDEB revealed no side effects and led to reduced wound erythema and blistering (*142*). Initially, no serious immunological reactions were observed, and although anti-C7 antibodies were detectable in serum, none was shown to bind to the skin or exacerbate blistering (*143,144*). The development of human C7 protein trials was expected thereafter, although thus far additional possible toxicology concerns have stalled clinical application, and further research will be required to assess the efficacy and safety of this therapy before clinical testing in patients with RDEB.

9. The Future

There is an urgent need for curative therapies for genetic disorders like RDEB that carry significant morbidity and mortality. In future, optimal treatment of RDEB will most likely involve combinations of drug, small molecule, gene, cell and protein therapies, with the collective ambition of reducing disease burden and compensating for, or repairing, the inherent skin pathology underscoring the blistering.

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