Progress in Epidermolysis Bullosa Research: Summary of DEBRA International Research Conference 2012

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Epidermolysis bullosa (EB), a group of heritable skin fragility disorders, is characterized by blistering, erosions, and chronic ulcers of the skin and mucous membranes, associated with extracutaneous manifestations with considerable morbidity and mortality. Mutations in as many as 18 distinct genes are known to underlie different EB-like disorders. The progress in molecular genetics of this group of disorders has improved the accuracy of diagnostic subclassification and prognostication, and has formed the basis for prenatal and preimplantation genetic diagnosis. Quite recently, inroads have also been made toward the treatment of EB by gene therapy, protein replacement, and cell-based approaches. DEBRA International, the premiere patient-advocacy organization, sponsored the Triennial Research Conference in November 2012 in Marbella, Spain[*]. This Conference Report summarizes the presentations and discussions of this meeting, with emphasis on the most recent progress in EB research over the past 3 years.

Introduction

EB, a group of disorders characterized by excessive fragility of the skin, in association with a number of extracutaneous manifestations, presents with blistering and erosions with considerable morbidity and mortality (Fine et al., 2008; Fine and Mellerio, 2009). EB is clearly a rare disease, as defined by fewer than 200,000 patients in the United States; less than 5 patients per 10,000 inhabitants in the European Union; and fewer than 50,000 affected individuals in Japan. Nevertheless, the estimated incidence of ~1:20,000 implies that there are as many as 30,000 affected individuals in the United States, and over half a million patients worldwide with the diagnosis of EB (Uitto, 2012). EB has served as a prototype of heritable skin diseases in which significant progress has been made over the past few decades from a condition defined purely by clinical description to an entity with profound understanding of the molecular defects at the genomic level (Table 1). Most recently, this progress on EB has culminated in early clinical trials of different molecular approaches for treatment and potential cure (Uitto et al., 2012).

The leading EB researchers from laboratories and hospitals around the world convened by invitation in November 2012 at Marbella, Spain, to discuss the state of the art of EB research (Figure 1). The goals of this DEBRA 2012 Research Conference were as follows: (1) to review the prospects of fundamental EB research and identify challenges in the development of clinical solutions; (2) to identify unexplored opportunities from relevant research in complementary areas; and (3) to derive a community consensus on EB research and therapy development priorities.

The authors of this report were members of the organizing committee; Drs Leena Bruckner-Tuderman (Freiburg University) and Jouni Uitto (Thomas Jefferson University) served as the co-chairs of the conference, and the program, together with speakers and session chairs, is in Supplementary Table S1 online. The proceedings of this international research meeting are summarized here with emphasis on advances made since the previous triennial meeting in 2009 (Uitto et al., 2010).

New phenotypes, novel genes

EB was initially recognized as a distinct diagnostic entity over a century ago, and the ensuing decades witnessed the identification of diverse phenotypes, which resulted in suggestions that there are over 30 clinical variants (Gedde-Dahl, 1986) (Table 1). The molecular era of EB research was initiated in the early 1990s, and within a few years as many as 10 distinct genes were shown to harbor mutations in the classic forms of EB (Table 2). More recently, a number of additional conditions manifesting with fragility of the skin, often in combination with extracutaneous findings, have been included in the spectrum of EB (Table 3). Thus, the total number of genes harboring mutations in various diseases in the spectrum of EB is now at least 18.

Model systems to study EB

The understanding of the pathomechanisms has been greatly facilitated by a number of model systems for various forms of EB. Of particular value have been genetically engineered mouse models and identification of spontaneous

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EB phenotypes both in domestic and wild animals. Since the last update (Bruckner-Tuderman *et al.*, 2010), several new animal models have been developed. For example, new mouse models for junctional EB (JEB) with reduced expression of the laminin γ2 chain (Bubier *et al.*, 2010; Adair-Kirk *et al.*, 2012) bring significant advantages over the existing knockout models for Herlitz JEB, which have been of limited use because of early lethality (Meng *et al.*, 2003). In one of them, a spontaneous mutation in the *Lamc2* gene generates a hypomorphic allele and low levels of laminin-332 at the basement membrane zone (Bubier *et al.*, 2010). The mutant mice survive and exhibit progressive skin blistering and extracutaneous features of variable severity; ultrafine recombination mapping unambiguously identified type

XVII collagen as a major genetic modifier of the strength of dermal–epidermal adhesion and provided an example of how naturally occurring genetic variation can act in epistasis to impact the severity of JEB (D. Roopenian, poster presentation in EB2012).

In another model, a doxycycline-controllable human laminin γ2 transgene was expressed in mice on the *Lamc2* knockout background (Adair-Kirk *et al.*, 2012). As long as doxycycline was included in the diet, the expression of human laminin-332 rescued the phenotype, but when doxycycline was withdrawn the mice developed trauma-induced skin blistering similar to human JEB. This mouse model will be valuable for testing novel biologically valid therapies for JEB.

In DEB, a clinical understanding of disease mechanisms has also been

advanced by new animal models. For example, clinical observations of chronic wounds in recessive dystrophic EB (RDEB) have suggested delayed wound closure, but no experimental data for this have existed so far. Controlled studies on wound healing using two different mouse models, the type VII collagen hypomorph and tamoxifen-mediated inactivation of the *Col7a1* gene, demonstrated that the loss of type VII collagen impairs early wound healing. Analysis of the dental phenotype in a mouse expressing human type VII collagen on the $Col7a1^{-/-}$ background (Umemoto *et al.*, 2012) revealed that the loss of type VII collagen hampers differentiation of ameloblasts and leads to defective enamel formation, which can be corrected by expressing human type VII collagen in the *Col7a1*-deficient mouse.

A spontaneous RDEB in Golden retriever dogs results from the homozygous *Col7a1* mutation p.G1906S (Gache *et al.*, 2011). Affected pups exhibit cutaneous and mucosal blistering, scarring, and dystrophy, as well as loss of nails. This relatively large animal model has been useful for testing the correction of dermal–epidermal dysadhesion using genetically modified epidermal grafts (Gache *et al.*, 2011) and for testing protein replacement therapy.

Recently, the first animal model for dominant DEB was discovered. Rats carrying a spontaneous heterozygous glycine substitution mutation in

Table 1. Decades of diagnostics and research on EB with selected milestones

Decades	Milestones and achievements
1880s	Recognition of EB as a clinically distinct entity
1900–1950s	Expansion of the clinical subsets by recognition of defined phenotypes
1960s	Recognition of three major subtypes—simplex, junctional, and dystrophic
1970–1980s	Early biochemical observations on disease pathomechanisms
1990s	Identification of genetic mutations in candidate genes with diagnostic implications, establishment of DNA-based prenatal testing, and preimplantation genetic diagnosis
2000s	Development of preclinical model systems and early proof-of-principle clinical trials
2010s	Implementation of phase I/II clinical trials for EB

Abbreviation: EB, epidermolysis bullosa.

Figure 1. Participants of the DEBRA International Research Conference 2012 in Marbella, Spain.

type VII collagen develop fragile and blister-prone skin as a consequence of functional abnormalities of the anchoring fibrils. The model recapitulates the features of human DDEB but, as in patients, the animals exhibit individual variations in the disease manifestations (Nyström *et al.*, 2013). Thus, this model may be well suited to analyze the effects of modifier genes in DEB.

Pathobiological disease mechanisms
In vitro cell culture systems have also been valuable for unraveling pathobiological disease mechanisms in EB. Intriguing novel evidence continues to emerge to indicate that the abundance of type VII collagen is not the only determinant of disease severity in DEB, but a host of other differentially expressed genes contribute to the phenotypes. For example, disease proteomics using normal and RDEB fibroblasts showed that the loss of type VII collagen affects a number of other proteins involved in the maintenance of basement membranes and the extracellular matrix in the skin (Küttner *et al.*, 2013). In this context, an interesting phenotypic modification was observed in monozygotic twins with RDEB and with identical *COL7A1* mutations, but with very different clinical manifestations. Several genes associated with the inhibition of TGF-β pathways were found to be differentially expressed in dermal fibroblasts of these twins, and are likely to explain the variable disease severity in the twins (Di Salvio *et al.*, 2012).

An *in vitro* skin tumor model constructed with *COL7A1* short-hairpin RNA-treated keratinocytes indicated that the loss of type VII collagen leads to enhanced angiogenesis, as evidenced by increased vascular endothelial growth factor and thrombospondin expression in the dermal compartment (Martins *et al.*, 2012). The activation of stromal fibroblasts is controlled by JAK signaling, and the cancer-associated fibroblast-induced tumor invasion by collaboration of JAK and ROCK signaling (Sanz-Moreno *et al.*, 2011), suggesting that inhibitors of these pathways could have potential as anticancer agents in RDEB (C. Gaggioli, personal communication).

A fibrin-based bioengineered human skin is another intriguing *in vitro/in vivo* model for understanding disease mechanisms and for testing novel therapies (Carretero *et al.*, 2011). It can be regenerated by orthotopic grafting onto the back of immunodeficient mice. Using patients' keratinocytes and fibroblasts, the system has permitted modeling of EB and other monogenic and acquired skin diseases. In addition, various gene and cell therapy approaches for *ex vivo* correction of cells have proved effective in this model, which holds promise for optimized testing of new therapeutic approaches (Carretero *et al.*, 2011).

Squamous cell carcinoma as a complication of EB
Squamous cell carcinomas (SCCs) represent the major cause of mortality in individuals with RDEB (>90% by the age of 55 years; Fine *et al.*, 2009). Thus, there remains a critical need to improve the understanding of the pathomechanisms of SCC in RDEB, as well as to develop novel diagnostic biomarkers and therapeutic approaches. To that end, some new findings on the pathophysiology of SCC in RDEB have emerged from studies focusing on both DNA mutations in keratinocytes and the influence of RDEB SCC–associated fibroblasts.

Recently, cutaneous SCCs have been shown to harbor the highest relative burden of DNA mutations among all

Table 2. The classic variants of EB

Category	Level of blistering/ mutated genes
Simplex	Basal cells
EBS	*KRT5, KRT14*
EBS-PA	*PLEC*
EBS-MD	*PLEC*
Junctional	Lamina lucida
HJEB, NHJEB	*LAMA3, LAMB3, LAMC2*
NHJEB	*BPAG2/COL17A1*
JEB-PA	*ITGA6, ITGB4*
Dystrophic	Sub-lamina densa
DDEB	*COL7A1*
RDEB	*COL7A1*

Abbreviations: DDEB, dominant dystrophic EB; EB, epidermolysis bullosa; EBS, EB simplex; EBS-MD, EBS with muscular dystrophy; EBS-PA, EBS with pyloric atresia; HJEB, Herlitz junctional EB; NHJEB, non-Herlitz junctional EB; RDEB, recessive dystrophic EB.

Table 3. Rare variants of EB-like blistering disorders with known gene defects

Clinical entity[1]	Level of blistering	Mutated genes/proteins	Reference
Ectodermal dysplasia–skin fragility syndrome	Suprabasal	*PKP1*/plakophilin 1	(McGrath *et al.*, 1997)
Kindler syndrome	Mixed	*KIND1*/kindlin 1	(Jobard *et al.*, 2003; Siegel *et al.*, 2003)
Laryngo–onycho–cutaneous syndrome	Lamina lucida	*LAMA3* A (LAMA3A)/laminin α3a	(McLean *et al.*, 2003)
Lethal acantholytic EB	Suprabasal	*DSP*/desmoplakin	(Jonkman *et al.*, 2005)
EBS, other	Basal keratinocytes	*DST*/BPAG1 (epithelial isoform)	(Groves *et al.*, 2010)
Acral peeling skin syndrome	Suprabasal	*TGM5*/transglutaminase-5	(Kiritsi *et al.*, 2010)
Blistering ± cardiomyopathy	Suprabasal	*JUP*/plakoglobin	(Pigors *et al.*, 2011)
EB-congenital nephrotic syndrome-interstitial lung disease	Mixed	*ITGA3*/integrin α3	(Has *et al.*, 2012)
Trauma-induced skin blistering (AR)	Lower epidermis	*EXPH5*/exophilin-5 (Slac2-b)	(McGrath *et al.*, 2012)

Abbreviations: EB, epidermolysis bullosa; EBS, EB simplex.
[1]The inheritance of these conditions is autosomal recessive.

human cancers, with >75% of non-RDEB tumors containing mutations in *NOTCH1* or *NOTCH2* (Wang *et al.*, 2011). The same study also identified *NOTCH1* mutations in two of three RDEB SCCs, indicating that these may be shared findings in RDEB as well as in common UV irradiation–associated SCCs in the general population. Further research has also shown a lack of *RAS* mutations in SCC in RDEB (0/6 samples) (Pourreyron *et al.*, 2007), but the presence of some mutations in *TP53* (3/8 SCCs) and *CDKN2A* promoter methylation (2/8 SCCs) has been noted (Arbiser *et al.*, 2004). Thus, RDEB SCCs and UV-associated SCCs appear to be genetically similar.

To identify therapeutic targets in SCC, an integrated approach to gene expression profiling beginning with primary keratinocytes in culture was developed (Watt *et al.*, 2011). The study established candidate drivers of SCCs by first defining a set of *in vitro* cancer genes and then comparing their expression in a range of clinical data sets that included normal skin, cutaneous SCCs, and psoriasis. This analysis revealed 21 upregulated genes that were then tested using a small interfering RNA screen to see which of the targets had the capacity to reduce xenograft tumor volume *in vivo*. Small-molecule inhibitors for one of the targets, polo-like kinase-1, showed efficacy in SCC. It is noteworthy that polo-like kinase-1 inhibitors are already in clinical trial for other malignancies, thereby presenting a possible direct translational research opportunity for EB SCCs. Small interfering RNA–mediated knockdown of one other target overexpressed in SCCs, C20orf20, induced apoptosis *in vitro* and reduced SCC growth *in vivo*. Thus, this study identified putative drivers of cutaneous SCCs as potential new therapeutic targets in treating this malignancy.

On comparing cultured fibroblasts isolated from RDEB individuals without SCC and fibroblasts directly from tumor matrix in RDEB and non-RDEB samples, it was shown that, although gene expression of RDEB-normal skin fibroblasts resembled that of UV SCC–associated fibroblasts, RDEB SCC–associated fibroblasts had a distinct gene expression profile, with many of the differentially expressed genes being involved in matrix and cell adhesion (Ng *et al.*, 2012). Collectively, these observations suggested that the matrix composition in RDEB skin forms a permissive environment for tumor development. In line with these observations, the combination of human or murine RDEB fibroblasts with SCC cells in a skin-equivalent culture model enhanced tumor invasiveness, and *in vivo* validation of these findings was derived from chemical tumor induction in the *Col7a1* hypomorphic mice that formed more aggressive SCCs than control mice (V. Mittapalli, personal communication).

Molecular therapies for EB

Gene therapy. To date, only one proof-of-principle report has been published (Mavilio *et al.*, 2006). This study involved the use of a retroviral vector to insert the *LAMB3* transgene into autologous keratinocytes. Now with over 6 years of follow-up, the graft reportedly continues to express laminin-332 at the dermal–epidermal junction, and the skin remains mechanically strong (De Luca *et al.*, 2009). Additional proof-of-principle studies for somatic gene therapy in individuals with RDEB are anticipated, but they are still at preclinical or early clinical phases. It should be noted that this kind of *ex vivo* keratinocyte grafting approach to gene therapy requires introduction of the transgene, often using viral vectors (Titeux *et al.*, 2010). This step can be bypassed by using keratinocytes derived from the unaffected skin of patients with revertant mosaicism, a natural gene therapy encountered in different forms of EB (Lai-Cheong *et al.*, 2011; Pasmooij and Jonkman, 2012). Finally, the *ex vivo* gene therapy could use keratinocytes differentiated from induced pluripotent stem cells derived from the patient's own cells, but clinical applications using this approach are yet to be reported (Itoh *et al.*, 2011; Tolar *et al.*, 2013).

Cell-based therapies. In contrast to gene therapy for EB, clinical trials of cell therapy have advanced further. Wong *et al.* (2008) demonstrated that a single intradermal injection of allo-geneic fibroblasts into unblistered RDEB skin can lead to the upregulation of *COL7A1* gene expression for 3–6 months, as well as increased type VII collagen protein for 9–12 months. The putative mechanism was postulated to be an increase in mutant, but partially functional, type VII collagen through upregulation of heparin-binding epidermal growth factor–like growth factor (Nagy *et al.*, 2011). However, whether allogeneic fibroblasts have the potential to improve the healing of chronic wounds in RDEB has not yet been reported in detail in placebo-controlled trials. Clinical assessment of intradermally injected allogeneic bone marrow–derived mesenchymal stromal cells, however, has been shown to improve wound healing in RDEB with *de novo* synthesis of type VII collagen. In this case, the clinical improvements after a single injection lasted for 4 months (Conget *et al.*, 2010).

Systemic cell therapy for RDEB has been assessed in a clinical trial of bone marrow transplantation in seven individuals with RDEB (Wagner *et al.*, 2010). On the basis of initial murine studies (Tolar *et al.*, 2009), this study used high-dose chemotherapy to immuno-myeloablate individuals with RDEB to allow lymphohematopoietic engraftment using unfiltered bone marrow to preserve all the hematogenous cell populations. It should be noted that this procedure carries a high risk of morbidity and significant mortality, one of the patients dying in this study from complications before grafting. Of the six subjects who received the infusion of bone marrow–derived stem cells, all demonstrated skin and marrow chimerism, and five individuals showed increased type VII collagen deposition at the dermal–epidermal junction. All had some degree of clinical benefit, including the patient in whom there was no change in type VII collagen, and improvement that has been maintained in a follow-up of up to 4 years post bone marrow transplantation (Tolar and Wagner, 2012). Although the precise nature of the bone marrow cells and paracrine factors underpinning the clinical responses remain to be determined, further clinical trials are in progress in a bid to minimize toxicity

(e.g., reduced intensity conditioning and additional infusion of mesenchymal stromal cells) and to explore whether other forms of EB, including Herlitz and non-Herlitz JEB, might also show clinicopathologic improvement following bone marrow transplantation.

Protein replacement. Another innovative approach to counteract blistering in EB involves protein replacement therapy. The initial preclinical studies used $Col7a1^{-/-}$ mice as a platform and demonstrated that the injection of purified human type VII collagen into these RDEB mice results in the formation of anchoring fibrils and correction of the blistering phenotype (Woodley et al., 2004; Remington et al., 2009). In subsequent studies, intravenous injection of recombinant type VII collagen in $Col7a1^{-/-}$ mice was shown to home into the wounds and restore type VII collagen expression and function (Woodley et al., 2013). In a model consisting of full-thickness skin wounds created in athymic mice, intravenously injected human type VII collagen homed to wound sites and incorporated into the dermal–epidermal junction at 2 weeks after injection. These data suggested that protein replacement therapy by intravenous injection of type VII collagen could restore the dermal–epidermal adhesion in RDEB by restoring anchoring fibril formation.

On the basis of the preclinical studies using various mouse models as a platform, a company, Lotus Tissue Repair (launched in 2011 and recently acquired by Shire Pharmaceuticals), has initiated a program with an aggressive timeline to commercialize the clinical application of recombinant type VII collagen for the treatment of RDEB (M. de Souza, personal communication). The company is currently performing extended animal studies using a RDEB dog model as target, and phase I clinical trials with FDA approval for intradermal and intravenous delivery are expected to follow soon. Although the prospects of protein therapy for RDEB look promising, careful attention needs to be placed on monitoring the side effects, such as development of antibodies to type VII collagen (de Souza and Rangel Miller, 2012).

The role of DEBRA International as a patient-advocacy organization

DEBRA International (http://www. DEBRA-international.org)–sponsored Triennial Research Conferences, such as EB2012, serve not only as a yardstick for research progress, but also to bring together the international EB research community to foster new ideas and collaborations and to set research priorities. DEBRA International is the patient-advocacy organization working on behalf of all patients with EB and their families worldwide, providing patient-support services, and funding both basic and clinical EB research. Patient-advocacy organizations are increasingly recognized for their role in setting the agenda for rare-disease therapy development, as exemplified by EB (Terry et al., 2007; de Souza and Rangel Miller, 2012). They can provide disease-, patient-, and socioeconomic-impact metrics, as well as direct access, to both patient populations and clinical specialists through clinical networks.

Numerous EB clinical research groups are developing disease severity scores and quality of life measures. DEBRA brought these groups together in a Workshop at EB2012 to derive consensus and identify gaps requiring further work. DEBRA is also supporting studies to document the natural history of EB, and validated clinical end points appropriate for therapy evaluation, and is engaging in studies to document the socioeconomic impact for patients with EB and their families.

The patients' perspective

At previous EB research conferences, positive feedback to patients' presentations on their experiences in clinical trials encouraged organizers to convene a ''Patient Forum'' at EB2012. Iñigo Ibarrondo, a patient whose parents founded DEBRA España, noted that it is sometimes difficult to grasp the purpose of lengthy investigations into apparently esoteric details when life is a daily struggle for those whose ''time is running out'', but that speaking directly with researchers was valuable for understanding how basic research could benefit the life of an EB patient. Also acknowledged was the complexity of the condition, with competition for attention and resources with other equally devastating diseases. The long, hard path of research was recognized as needing sometimes ''superhuman'' efforts, matching that of patients in their daily lives.

''You are walking and building a path for people with EB, away from death or struggling for life….Bottom line, thanks not only for walking this path with us, but also for us. Please keep walking.'' (Iñigo Ibarrondo, EB Patient Forum, EB2012).

There was a strong message from Iñigo and the patient group that ''avoiding unnecessary delay'' is urgent, but that participation at EB2012 had engendered optimism that progress is being made in taking diverse EB therapies into clinical trials.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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