Journal of Pathology
J Pathol 2011; **225:** 401–413
Published online in Wiley Online Library
(wileyonlinelibrary.com) DOI: 10.1002/path.2973

ORIGINAL PAPER

Overexpression of the *Flii* gene increases dermal–epidermal blistering in an autoimmune ColVII mouse model of epidermolysis bullosa acquisita

Zlatko Kopecki,[1,2] Ruth M Arkell,[3] Xanthe L Strudwick,[1] Misa Hirose,[4] Ralf J Ludwig,[4] Johannes S Kern,[5] Leena Bruckner-Tuderman,[5] Detlef Zillikens,[4] Dedee F Murrell[6] and Allison J Cowin[1,2,7]*

[1] *Women's and Children's Health Research Institute, 72 King William Road, North Adelaide, SA, Australia*
[2] *Discipline of Paediatrics, University of Adelaide, SA, Australia*
[3] *Research School of Biological Sciences, Australian National University, ACT, Australia*
[4] *Department of Dermatology, University of Lubeck, Germany*
[5] *Department of Dermatology, University Medical Center Freiburg, Germany and Freiburg Institute for Advanced Studies, School of Lifesciences/Lifenet, Germany*
[6] *Department of Dermatology, St George Hospital and University of NSW, Sydney, Australia*
[7] *School of Pharmacy and Medical Sciences, University of South Australia, Australia*

**Correspondence to: Allison J Cowin, Women's and Children's Health Research Institute, 72 King William Road, North Adelaide, Australia.*
e-mail: allison.cowin@adelaide.edu.au

Abstract

Epidermolysis bullosa (EB) is a severe genetic skin fragility syndrome characterized by blister formation. The molecular basis of EB is still largely unknown and wound healing in patients suffering from EB remains a major challenge to their survival. Our previous studies have identified the actin remodelling protein Flightless I (Flii) as an important mediator of wound repair. Here we identify Flii as a novel target involved in skin blistering. Flii expression was significantly elevated in 30 patients with EB, most prominently in patients with recessive dystrophic EB (RDEB) who have defects in production of type VII collagen (ColVII). Using an autoimmune ColVII murine model of EB acquisita (EBA) and an immunocompetent-ColVII-hypomorphic genetic mouse model of RDEB together with murine *Flii* alleles, we investigated the contribution of *Flii* to EB. Overexpression of *Flii* produced severe blistering post-induction of EBA, while decreased *Flii* reduced blister severity, elevated integrin expression, and improved ColVII production. $Flii^{+/-}$ blistered skin showed reduced α-SMA, TGF-β1, and Smad 2/3 expression, suggesting that decreasing Flii may affect fibrosis. In support of this, *Flii*-deficient fibroblasts from EBA mice were less able to contract collagen gels *in vitro*; however, addition of TGF-β1 restored collagen contraction, suggesting an interplay between Flii and TGF-β1. Elevated *Flii* gene and protein expression was further observed in the blisters of ColVII hypomorphic mice, a murine model of RDEB, suggesting that reducing Flii in blistered skin could be a potential new approach for treating patients with EB.

Keywords: Flightless; epidermolysis bullosa; wound healing; type VII collagen; Flii; TGF-β

Received 15 June 2011; Accepted 18 July 2011

No conflicts of interest were declared.

Introduction

EB comprises a group of inherited disorders characterized by blistering of the skin and mucosal surfaces. Wound healing in patients suffering from EB poses a major challenge to their survival [1–3]. An altered structure of hemidesmosomes, type VII collagen (ColVII) anchoring fibrils, and expression of integrin receptors and proteins involved in mediating skin adhesion all contribute to the blistering observed in EB patients [4,5]. Classification of different genetic subtypes is based on the mode of inheritance and clinical and pathological findings [6,7]. The three main types of EB include EB simplex (EBS), characterized by the fragility of basal keratinocytes; junctional EB (JEB), characterized by tissue separation within the lamina lucida; and dystrophic EB (DEB), characterized by blister formation at the level of ColVII anchoring fibrils [8]. EB acquisita (EBA) is an acquired blistering disease of skin classified by sub-epidermal blisters and tissue-bound autoantibodies against the ColVII anchoring fibrils, with similar clinical features to those observed in DEB patients [9–11]. Current treatments for EB are symptomatic and do not change the course of the disease, while gene therapy approaches have shown limited success and numerous technical and safety problems [6,12–14]. Additionally, EBA is highly resistant to immunosuppressive

therapy, with little evidence of efficacious treatments [15].

The actin remodelling protein Flii has an important role in mediating cellular adhesion, hemidesmosome structure, and integrin signalling [16]. Flii is a member of the gelsolin family of proteins that regulate actin by severing pre-existing filaments and/or capping filament ends to enable filament reassembly into new cytoskeletal structures [17]. Flii has a unique structure with homology to two gene families, the gelsolin superfamily and leucine-rich repeat (LRR)-containing proteins [18–20]. It is expressed in both the epidermis and the dermis; has the ability to translocate from the cytoplasm to the nucleus; and is also a secreted protein [20–22]. In addition, Flii is expressed at sites of focal adhesion and migratory structures involved in cellular motility [23]. Flii is also a transcriptional co-activator [24] which competes with the β-catenin and FLAP1 activators of Tcf/LEF-dependent genes [25]. The diversity of this molecule and its functions are also evident in recent reports suggesting that *Flii* may play a prominent role in signalling networks involved in the innate immune system [26–28], while the loss of *Flii* gene function in both *Drosophila* and the mouse leads to early embryonic lethality [19,29].

We have previously shown that reducing *Flii* expression improves *in vivo* wound repair, while *Flii* overexpression results in delayed wound closure, increased skin fragility, and impaired wound healing [16,22]. Flii affects cellular motility and matrix production, and is a contributing factor to poor wound repair in incisional wounds, partial thickness burn wounds, and elderly skin [21,22,30]. Here we have investigated the expression of Flii in the blisters of patients with different EB syndromes and have further investigated the function of Flii in EB using an autoimmune ColVII murine model of EBA and an immunocompetent genetic ColVII hypomorphic mouse model of RDEB together with murine *Flii* alleles to investigate the contribution of Flii to the pathogenesis of EB.

Materials and methods

Human tissue samples

Skin biopsies from 30 EB patients (specifically, EB simplex $n = 8$, junctional EB $n = 12$, and dystrophic EB $n = 10$) were collected through the National EB Diagnostic Laboratory, St George Hospital, Sydney, Australia. The clinical investigations were conducted according to the Declaration of Helsinki principles and written informed consent was obtained. Classification of EB sub-types was based on clinical presentations, histological site of blister formation, and gene analysis. The control group consisted of human skin samples collected during cosmetic surgical procedures from four patients with no known dermatological condition.

Animal studies

Mice were maintained according to Australian Standards for Animal Care under protocols approved by The Australian National University Animal Ethics Committee and the Child, Youth and Women's Health Service Animal Ethics Committee. All strains were BALB/c congenic and were maintained as homozygous colonies or by continuous backcross to BALB/c animals. Wild-type controls were obtained from BALB/c inbred litters. The murine alleles of Flii used in this study were (1) $Flii^{tm1Hdc}$ (MGI:2 179 825), a targeted null allele of *Flii* [29]. The genotype of a heterozygous carrier of this allele is written as $Flii^{+/-}$; and (2) Tg(FLII)1Hdc (MGI:3 796 828), a transgenic strain expressing exogenous human *FLII* due to the insertion of the human cosmid clone c110H8 [29]. These animals carry two copies of the mouse *Flii* gene and two copies of the human *Flii* transgene ($Flii^{+/+}$; $Flii^{Tg/Tg}$), denoted throughout this article as $FLII^{Tg/Tg}$.

EBA was induced by injecting 3- to 4-week-old wild-type ($Flii^{+/+}$), $Flii^{+/-}$ or $FLII^{Tg/Tg}$ mice with rabbit anti-mouse ColVII antibody (0.3 mg/g body weight) subcutaneously every second day for 10 days [31]. Mice were examined daily for evidence of cutaneous lesions and the extent of skin disease, and were euthanized at day 16 post-initial injection when the number of blisters peaked. Control animals injected with normal rabbit IgG failed to develop skin blisters. Hypomorphic ColVII mice were generated on the C57BL/6 background as described previously [32]. Blistered skin from the front paws, non-blistered skin from the back, and inflamed skin from hind paws were collected from 3-week-old ColVII hypomorphic mice, and normal skin from the same anatomical sites of wild-type controls.

Floating collagen gel contraction (FCGC) assay

3D collagen gels were prepared as described previously [33]. Primary fibroblasts from $Flii^{+/-}$, wild-type, and $FLII^{Tg/Tg}$ EBA mouse skin (1×10^5 cells/ml) were mixed into the collagen gel added to 48-well flat-bottomed plates (500 µl per well) and allowed to set for 120 min at 37 °C and 5% CO_2. The gel was dislodged by the addition of 1× DMEM media. Contraction of the collagen gel was determined at 72 h by measuring the residual area of the gel using Image Pro-Plus software (MediaCybernetics Inc, MD, USA). Flii neutralizing antibody (50 µg/ml) [22] was added to the collagen gel with $FLII^{Tg/Tg}$ fibroblasts extracted from EBA mice. TGF-β1 (2 ng/ml) was added to the collagen gel with $Flii^{+/-}$ fibroblasts extracted from EBA mice.

Materials and methods for histology, immunohistochemistry, western blotting, cell adhesion and proliferation assays, RT-PCR, co-immunoprecipitation, and statistical analysis may be found in the Supporting information, Supplementary materials and methods.

Results

Flii is significantly increased in blistered skin of EB patients

Human skin biopsies of blistered and non-blistered lesions of patients suffering from EBS, JEB, and DEB were analysed in a blinded fashion for both Flii and gelsolin expression. Flii, but not family member gelsolin, was elevated in the blistered skin of patients of all three main EB types compared with both non-blistered patient skin and biopsy samples from control individuals (Figures 1A–1J). Flii staining was present both at the dermal–epidermal junction and at the sites of blister formation. Flii expression was evident in both the epidermis and the dermis of the vesicular skin lesions and was most pronounced in patients suffering from DEB (Figure 1H). Quantification of Flii fluorescence intensity in different subtypes of EB, namely EBS (Figure 1K), JEB (Figure 1L), and DEB (Figure 1M), showed that Flii expression was increased in the majority of different EB subtypes. The highest expression of Flii was observed in blistered skin of patients with RDEB generalized, while patients with DDEB showed elevated Flii activity in both non-blistered and blistered patient skin compared with control sections (Figure 1M).

Flii-overexpressing mice exhibit severe blister formation

A mouse model of the autoimmune condition EBA which has phenotypic features of human DEB was used following methods described previously [31]. Multiple injections of ColVII antibody over a period of 16 days led to an increasing immune response to ColVII, resulting in skin disease with high numbers of blisters, erosive lesions, and crusts (Figure 2A). Mice with different levels of *Flii* gene expression ($Flii^{+/-}$, $Flii^{+/+}$, and $FLII^{Tg/Tg}$) were injected with rabbit anti-mouse ColVII antibody, which resulted in a varying degree of blister formation. While all groups of ColVII-treated mice developed EBA with more than 20% of the skin surface acquiring blister lesions at day 16 post-initial injection, macroscopically $Flii^{+/-}$ and wild-type EBA mice had less extensive blistering and a decreased number of lesions compared with overexpressing $FLII^{Tg/Tg}$ EBA mice (Figure 2A). Electron microscopy was used to confirm the greater degree of dermal–epidermal separation and the diffuse arrangement of ColVII anchoring fibrils in $FLII^{Tg/Tg}$ EBA mice compared with $Flii^{+/-}$ and wild-type EBA counterparts (Figures 2B–2G). Examination of the blister lesions using light microscopy revealed an improved dermal architecture in both $Flii^{+/-}$ and wild-type EBA mice skin compared with $FLII^{Tg/Tg}$ EBA mice lesions (Figure 2H) and smaller blister lesions in $Flii^{+/-}$ EBA mice compared with wild-type and $FLII^{Tg/Tg}$ counterparts (Figure 2H). Overexpression of *Flii* in $FLII^{Tg/Tg}$ EBA mice led to a disrupted dermal architecture, large blisters, and a significantly higher histological blister score compared with both $Flii^{+/-}$ and wild-type EBA mice (Figures 2H and 2I).

Flii expression is increased in blistered mouse skin and decreasing its expression improves ColVII expression

Flii expression was examined in skin sections of $Flii^{+/-}$, wild-type, and $FLII^{Tg/Tg}$ EBA-induced mice. Increased Flii expression was observed in both blistered and non-blistered sections of $Flii^{+/-}$, wild-type, and $FLII^{Tg/Tg}$ EBA mice compared with IgG controls (Figure 3A). $Flii^{+/-}$ EBA mice had significantly reduced Flii expression in blistered skin compared with wild-type and $FLII^{Tg/Tg}$ EBA mice, while $FLII^{Tg/Tg}$ EBA mice had significantly elevated Flii expression compared with wild-type mice (Figure 3B). Western blotting confirmed the immunofluorescent data, with Flii expression being increased in response to blistering in both wild-type (two-fold increase) and $FLII^{Tg/Tg}$ (three-fold increase) EBA mice skin (Figure 3C). Examination of the effect of *Flii* on ColVII production in unwounded skin revealed significantly decreased ColVII staining in $FLII^{Tg/Tg}$ mice, while *Flii*-deficient mice had improved ColVII production (Figures 3D and 3E).

Manipulation of *Flii* alters the integrin expression during skin blistering

Integrins are important adhesion receptors that mediate the cell–matrix adhesion and may be defective in JEB [34,35]. To determine whether *Flii* affected integrin receptors in blister lesions, the expression of integrin α3, α6, and β1 subunits was examined in skin of $Flii^{+/-}$, wild-type, and $FLII^{Tg/Tg}$ EBA mice. Both $Flii^{+/-}$ and wild-type EBA mice blisters showed strong expression of integrin α3 and β1 subunits, with staining throughout the epidermis, while $FLII^{Tg/Tg}$ EBA mice blisters had weak staining of integrin α3 and β1 subunits which was mainly concentrated on the apical part of the epidermis (Figures 4A–4L). Compared with wild-type IgG controls, integrin expression was increased in response to blistering; however, manipulation of *Flii* in blistered skin did not affect integrin α6 expression (Figures 4E–4H). Quantifying integrin α3, α6, and β1 fluorescence revealed significantly increased integrin expression in response to skin blistering in both $Flii^{+/-}$ and wild-type EBA blisters compared with non-blistered skin and IgG controls, and impaired up-regulation of integrin α3 and β1 expression in $FLII^{Tg/Tg}$ blister lesions (Figures 4M–4O). Increased integrin β1 expression in $Flii^{+/-}$ and wild-type EBA mice blisters was confirmed by western blotting (Figure 4P).

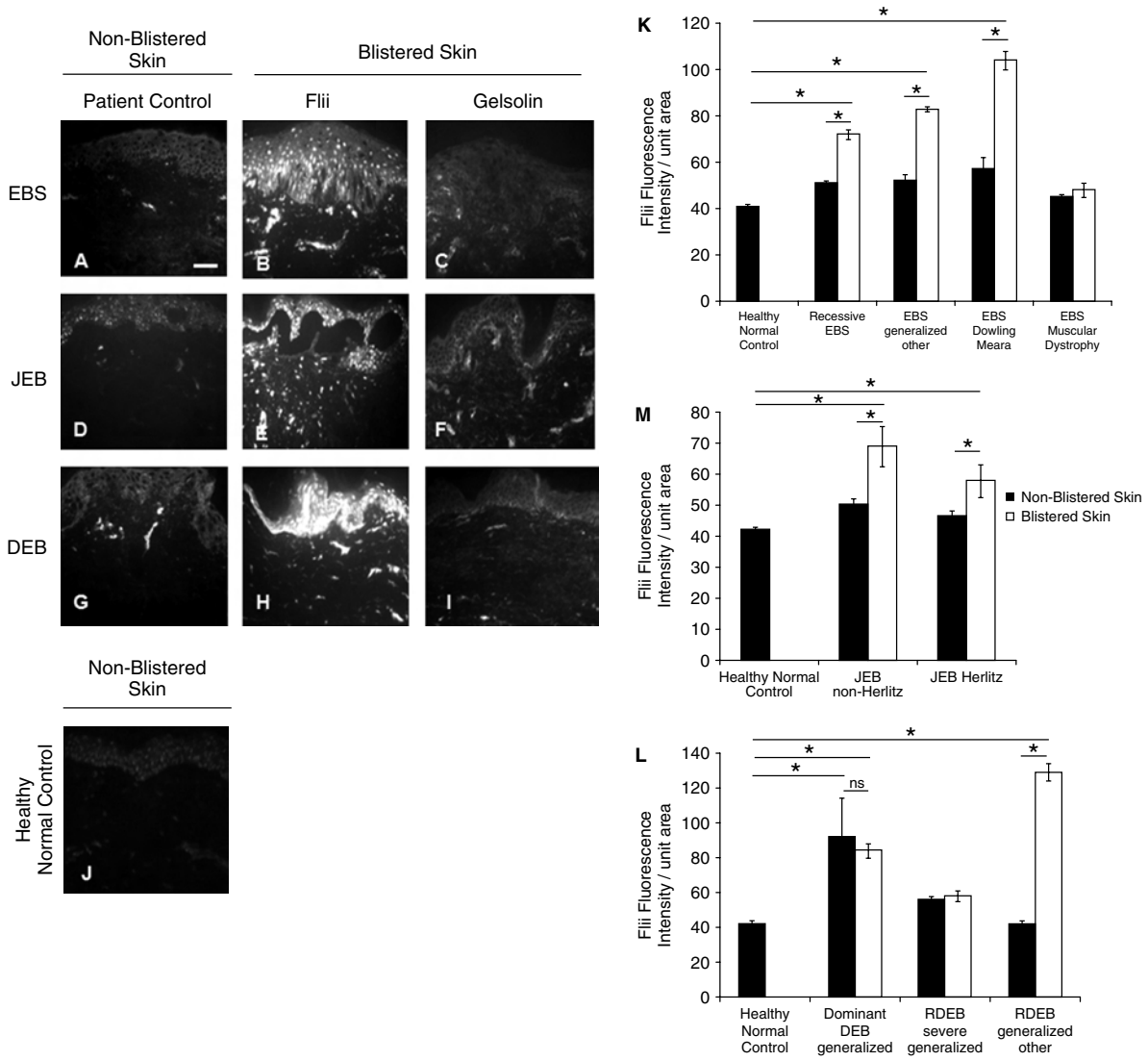

Figure 1. Flii, but not gelsolin, is significantly increased in response to blistering in human wounds of different EB types. (A–M) Representative images and graphical analysis of Flii and gelsolin expression in human samples of blistered and non-blistered skin from patients with different EB types ($n = 30$) and control non-blistered skin samples from healthy individuals ($n = 4$). Flii activity is specifically increased in response to skin blistering in EB simplex (B, K), junctional EB (E, L), and dystrophic EB (H, M) patients both at the dermal–epidermal junction and at the blister site compared with non-blistered patients' skin (A, D, G) or skin from healthy individuals (J). RDEB = recessive dystrophic EB. Scale bar in A = 200 μm. $n = 30$. Mean ± SEM. $^*p < 0.05$.

Reducing Flii expression improves the adhesion and proliferation of fibroblasts from EBA-induced mice

The adhesion properties of primary early passage fibroblasts and keratinocytes extracted from EBA-induced $Flii^{+/-}$, wild-type, and $FLII^{Tg/Tg}$ mice were investigated. No significant difference was observed in the adhesion of $Flii^{+/-}$, wild-type, and $FLII^{Tg/Tg}$ keratinocytes extracted from EBA-induced or IgG control mice (Supporting information, Supplementary Figure 1). However, significantly decreased adhesion of fibroblasts extracted from the skin of EBA-induced mice was observed compared with fibroblasts extracted from IgG control mice (Supporting information, Supplementary Figure 1). Additionally, $Flii^{+/-}$ fibroblasts from EBA-induced mice had improved adhesion compared with wild-type fibroblasts from EBA mice, while Flii overexpression led to significantly decreased fibroblast adhesion (Supporting information, Supplementary Figure 1). No significant difference in adhesion was observed with any of the extracellular matrix substrates investigated (Supporting information, Supplementary Figure 1). Exogenous addition of a Flii neutralizing antibody (FliiAb) to fibroblasts extracted from wild-type EBA-induced mice restored cellular adhesion and proliferation to those of normal fibroblasts extracted from non-induced controls (Figures 5A and 5B).

Flii is increased in response to blistering in ColVII hypomorphic mice

Genetically modified immunocompetent ColVII hypomorphic mice have 10% of normal ColVII levels and most closely present the severe phenotypical features of human RDEB, including mucocutaneous blistering,

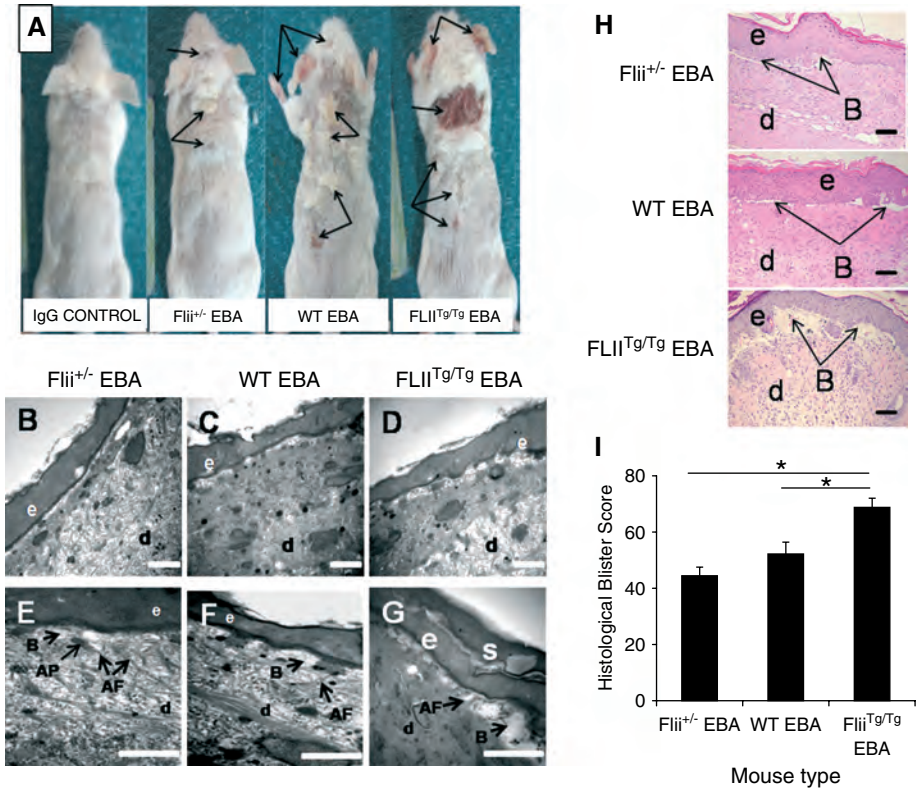

Figure 2. Effect of *Flii* gene expression on blister formation. (A) Skin blistering in $Flii^{+/-}$, WT, $FLII^{Tg/Tg}$ EBA, and IgG control mice 12 days post-initial injection showing lanced blisters and erosions on the back, ears, and paws (arrows) of mice. $FLII^{Tg/Tg}$ EBA mice develop more severe blisters than $Flii^{+/-}$ and WT EBA mice. (B–G) Transition electron microscopy shows more extensive dermal–epidermal separation in the skin of $FLII^{Tg/Tg}$ EBA mice, with diffuse arrangement of anchoring fibrils at the site of blister formation. e = epidermis; d = dermis; AF = anchoring fibrils; B = blister; AP = anchoring plaque; S = scab. Original magnification: ×13 000–25 000. Scale bar = 1 μm. $n = 2$. (H, I) Representative images of haematoxylin and eosin-stained blisters at day 16 post-initial injection and graphical analysis of the histological blister score. e = epidermis; B = blister; d = dermis. Original magnification: ×10. Scale bar = 500 μm. $n = 4$. Mean ± SEM. $^{*}p < 0.05$.

growth retardation, and pseudosyndactyly (Figure 6A). Studies have shown that the ColVII hypomorphic mice develop blistering on the back in response to mechanical trauma, high inflammation and extensive blistering, and pseudosyndactyly in the front paws [32]. Flii expression was increased in the epidermis, dermis, and around the blister site in the front paws of ColVII hypomorphic mice compared with wild-type counterparts (Figures 6B and 6C). Increased Flii expression was also observed in the hind paws of ColVII hypomorphic mice but no significant difference for Flii expression was observed between back skin of ColVII hypomorphic mice and wild-type control mice (Figure 6C). *Flii* gene expression was significantly up-regulated in blistered front paws of ColVII hypomorphic mice compared with non-blistered front paws of wild-type mice (Figure 6D).

Flii affects the TGF-β1/Smad signalling during skin blistering

TGF-β1 is an important contributor to excessive scarring and a major promoter of fibroblast differentiation into myofibroblasts. Repeated blistering in EB patients leads to excessive induction of tissue repair and up-regulation of TGF-β1, resulting in myofibroblast-generated contractile fibrosis and pseudosyndactyly [36]. To determine whether Flii modulation of TGF-β affected wound repair of blister lesions, both TGF-β1 and α-SMA expression was examined in $Flii^{+/-}$, wild-type, and $FLII^{Tg/Tg}$ EBA-induced mice. Flii-overexpressing EBA-induced mice had significantly increased TGF-β1 expression in both non-blistered and blistered skin in the deep dermis and at the site of blister formation compared with $Flii^{+/-}$ and wild-type EBA counterparts (Figures 7A and 7B). Conversely, $Flii^{+/-}$ EBA-induced blistered mouse wounds had significantly reduced TGF-β1 and α-SMA expression *in vivo* compared with wild-type and $FLII^{Tg/Tg}$ EBA-induced mice (Figures 7A–7C). The effect of *Flii* on TGF-β/Smad signalling was investigated using western blotting (Figures 7D–7G). $Flii^{+/-}$ EBA-induced blistered skin had reduced TGF-β1 and Smad 2/3 signalling, while *Flii*-overexpressing EBA-induced blistered skin had significantly elevated TGF-β1 and Smad 2/3 expression (Figures 7D–7F). To gain a mechanistic insight into the potential role of Flii in TGF-β signalling, EBA blistered skin tissue lysate was used to investigate possible Flii binding partners. Flii was found to associate with activating proteins (AP-1), cFos and cJun, in blistered skin (Figure 7G). Family member gelsolin, which is structurally similar to

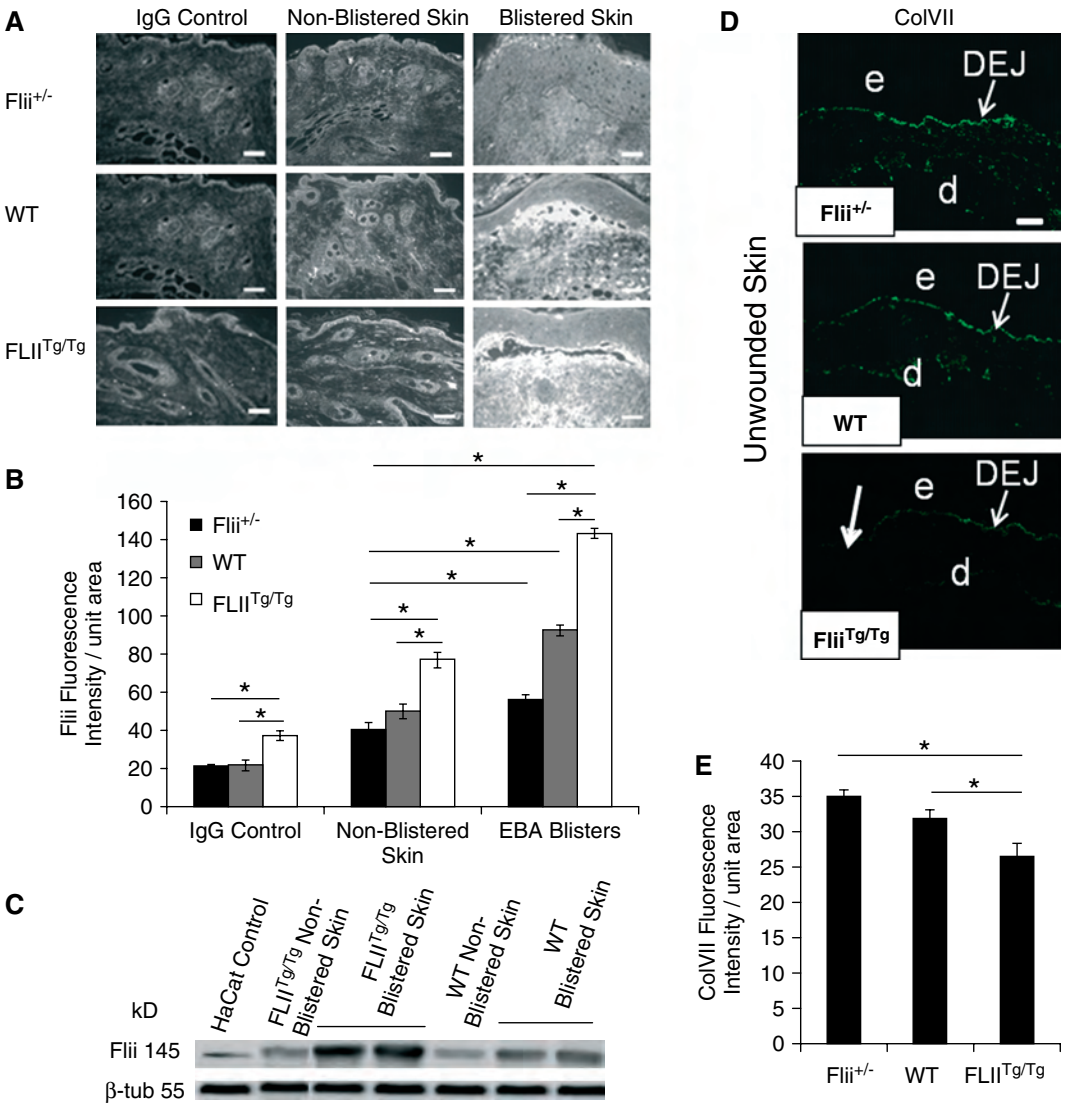

Figure 3. Flii is up-regulated in response to blistering in EBA mice. (A, B) Flii expression is increased in non-blistered and blistered skin of EBA mice. *Flii*$^{+/-}$ EBA mice have significantly reduced Flii expression in blistered skin compared with both WT and *FLII*$^{Tg/Tg}$ EBA mice. Original magnification: ×40. Scale bar in A = 100 μm. $n = 4$. (C) Representative western blot of Flii in WT and *FLII*$^{Tg/Tg}$ EBA mice showing increased Flii expression in response to skin blistering (two-fold increase WT and three-fold increase *FLII*$^{Tg/Tg}$ EBA). Data are representative of two independent repeats; fold increase was analysed based on the band intensity and normalized to the β-tubulin control. (D, E) Immunofluorescence for ColVII in *Flii*$^{+/-}$, WT, and *FLII*$^{Tg/Tg}$ unwounded skin shows areas of reduced ColVII in *FLII*$^{Tg/Tg}$ skin (arrow). DEJ = dermal–epidermal junction. Original magnification: ×40. Scale bar = 100 μm. $n = 6$. Mean ± SEM. $^{*}p < 0.05$.

Flii, was found not to bind to cFos and cJun, suggesting that the interaction with Flii is specific to this protein.

Flii requires TGF-β1 for fibroblast differentiation and collagen contraction

Incorporation of α-SMA into the actin cytoskeleton allows fibroblasts to exert enhanced contractile activity [37]. Reduced Flii expression in *Flii*$^{+/-}$ fibroblasts extracted from EBA-induced mice resulted in weak expression and decreased incorporation of α-SMA into stress fibres (Figure 8C). To determine the ability of *Flii*$^{+/-}$, wild-type, and *FLII*$^{Tg/Tg}$ fibroblasts extracted from EBA-induced mice to contract collagen, a fibroblast-populated collagen gel contraction assay was used. Wild-type and *FLII*$^{Tg/Tg}$ fibroblasts extracted from EBA-induced mice were able to contract the collagen gel, unlike *Flii*$^{+/-}$ fibroblasts extracted from EBA-induced mice (Figures 8A and 8B). Exogenous addition of TGF-β1 to the *Flii*-deficient fibroblasts extracted from EBA-induced mice in the collagen gel restored the contractile ability of the *Flii*$^{+/-}$ fibroblasts to a level similar to that of wild-type fibroblasts extracted from EBA-induced mice (Figures 8A and 8B). Conversely, when FliiAb was added to collagen gels populated with *FLII*$^{Tg/Tg}$ fibroblasts extracted from EBA-induced mice, collagen contraction was similar to that observed in *Flii*$^{+/-}$ fibroblasts extracted from EBA-induced mice (Figures 8A and 8B).

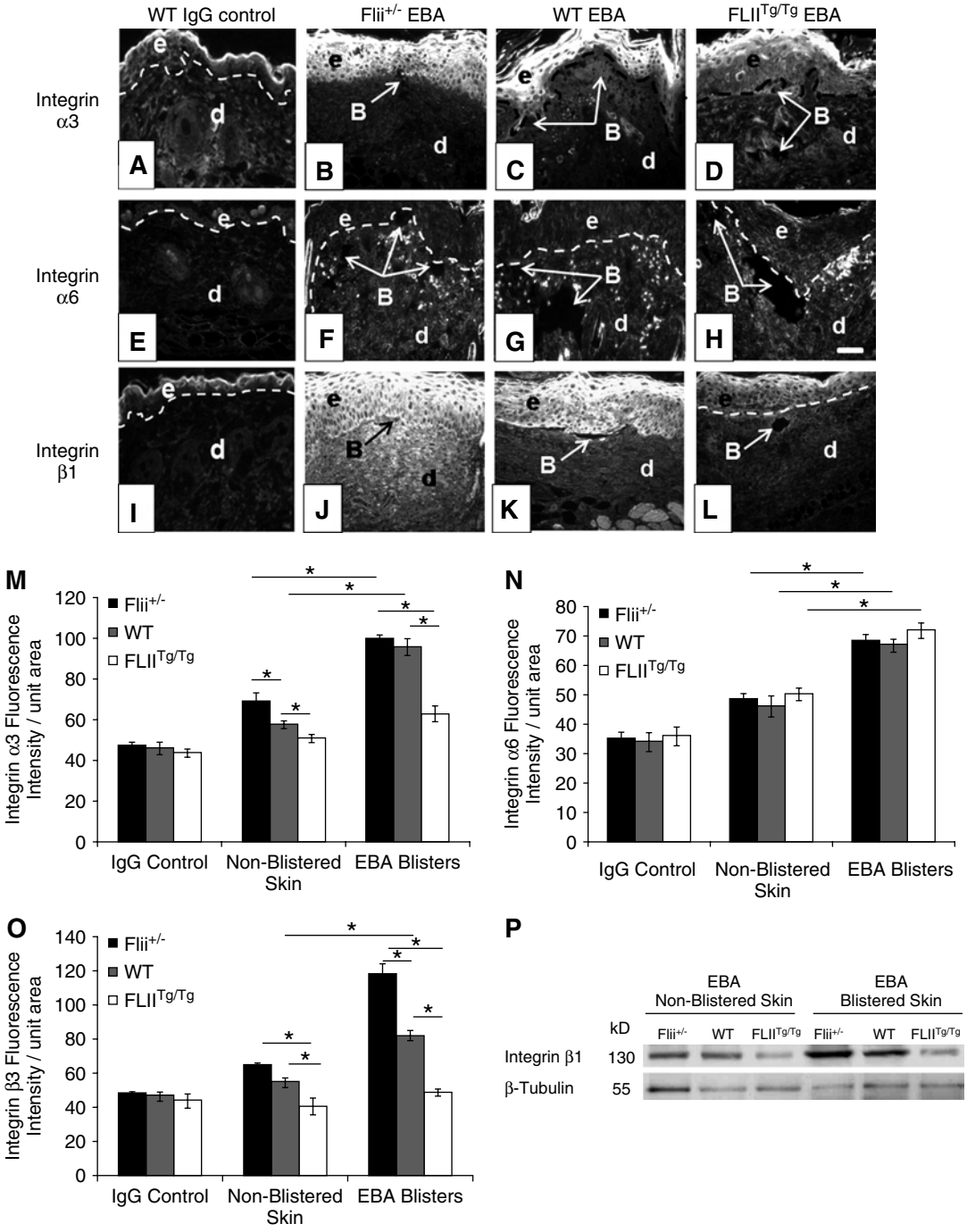

Figure 4. Effect of Flii on integrin expression in blister lesions. (A–O) Representative images and graphical analysis of integrin α3, α6, and β1 expression in blistered skin of *Flii*$^{+/-}$, WT, and *FLII*$^{Tg/Tg}$ EBA mice. No difference is observed in the expression of integrin α6 in EBA mice with different *Flii* levels. Reduced expression of integrin α3 and integrin β1 subunit is observed in the epidermis, dermis, and around the blister site in *FLII*$^{Tg/Tg}$ EBA mice when compared with *Flii*$^{+/-}$ and WT EBA counterparts. e = epidermis; d = dermis; B = blister; dotted line = dermal–epidermal junction. Original magnification: ×40. Scale bar in F = 100 μm. $n = 4$. Mean ± SEM. $^{*}p < 0.05$. (P) Representative western blot of integrin β1 in *Flii*$^{+/-}$, WT, and *FLII*$^{Tg/Tg}$ EBA mice showing increased integrin β1 expression in response to skin blistering. Unlike in *Flii*$^{+/-}$ and WT mice skin, integrin β1 is not up-regulated in skin of *FLII*$^{Tg/Tg}$ mice in response to blistering. Data are representative of two independent repeats; equal loading is demonstrated with the β-tubulin control.

Discussion

EB is a complex group of genetic disorders producing various degrees of recurrent skin blistering [38]. This study, in which both human samples and two different animal models of EB and EBA were used, is the first to present the involvement of the cytoskeletal protein Flii in EB and EBA. Previous studies have identified Flii as a negative regulator of wound healing [21,22]. Overexpression of *Flii* leads to impaired cellular adhesion, diffuse ColVII anchoring fibrils, and decreased skin tensile strength [16,20,22,30]. Modulation of Flii activity by either genetic knock-down or neutralizing antibodies improves wound healing in both

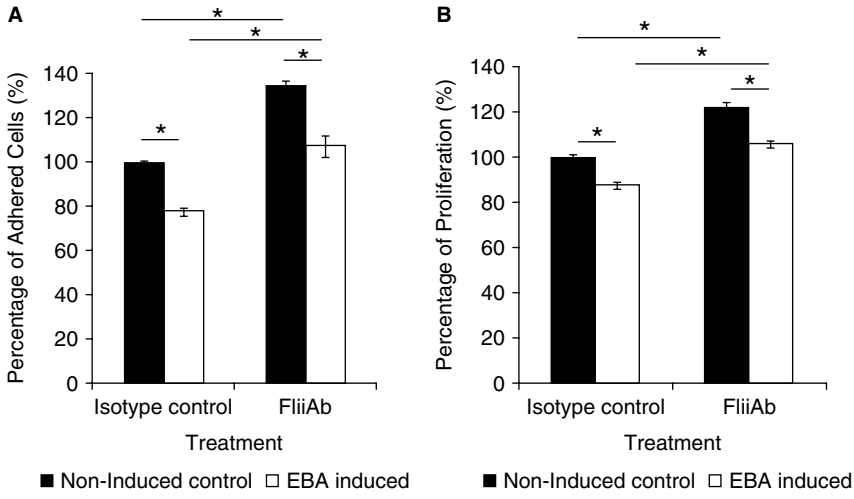

Figure 5. Application of Flii neutralizing antibodies improves adhesion and proliferation of EBA fibroblasts. (A, B) Primary fibroblasts extracted from WT EBA and IgG control mice treated with Flii neutralizing antibody (FliiAb) have significantly improved cellular adhesion and proliferation, similar to cells extracted from IgG control mice treated with isotype control antibody. Mean ± SEM. $n = 6$. All experiments were repeated in triplicate. $^{*}p < 0.05$.

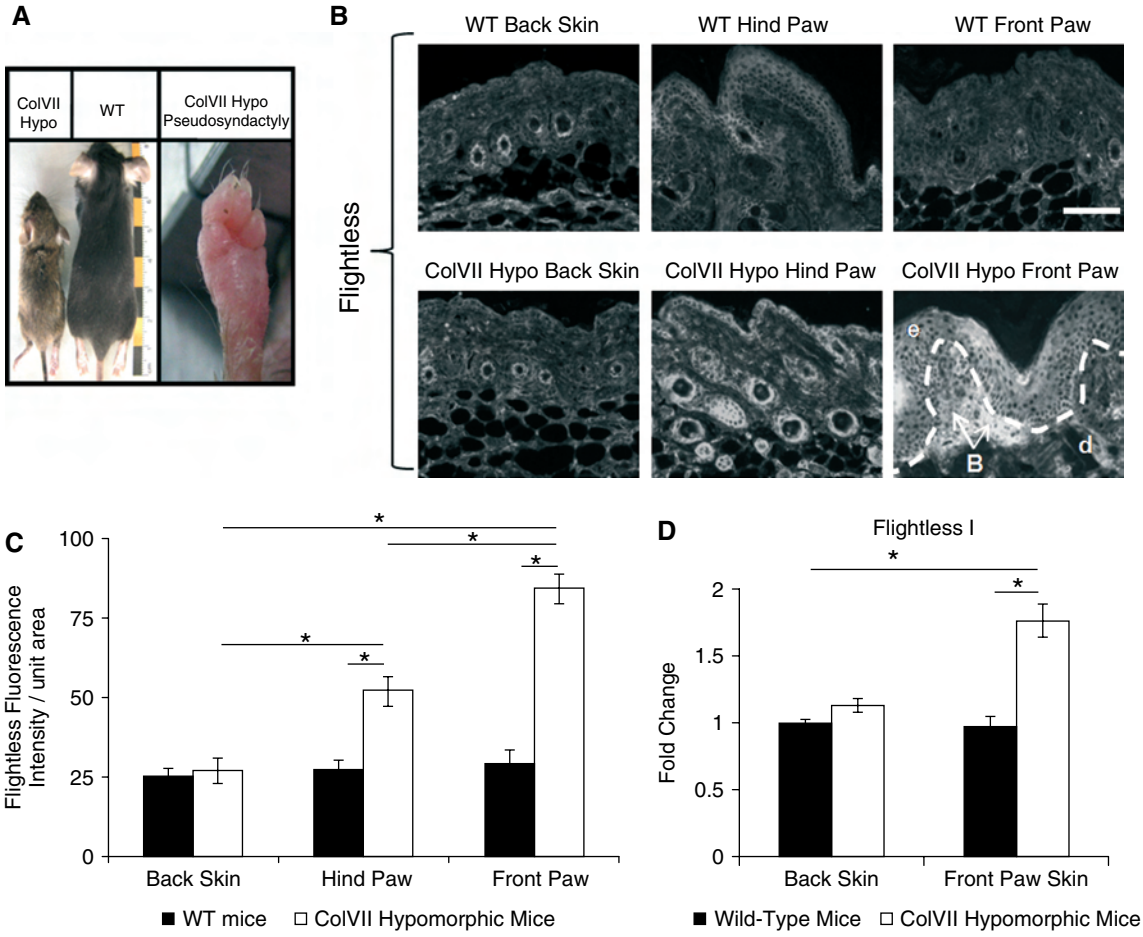

Figure 6. Flii expression is increased in blistered skin of ColVII hypomorphic mice. (A) ColVII hypomorphic mice have 10% of normal ColVII levels, resulting in mucocutaneous blistering, retarded growth, and pseudosyndactyly on the front paws, features clinically seen in patients suffering from DEB. (B–D) Representative images and graphical analysis of Flii protein and gene expression in response to blistering in ColVII hypomorphic mice. Flii protein and gene expression is significantly increased in blistered skin from paws compared with non-blistered back skin of ColVII hypomorphic mice and WT counterparts. Original magnification: ×40. e = epidermis; d = dermis; B = blister; dotted line = dermal–epidermal junction. Scale bar in B = 250 μm. $n = 4$. Mean ± SEM. $^{*}p < 0.05$.

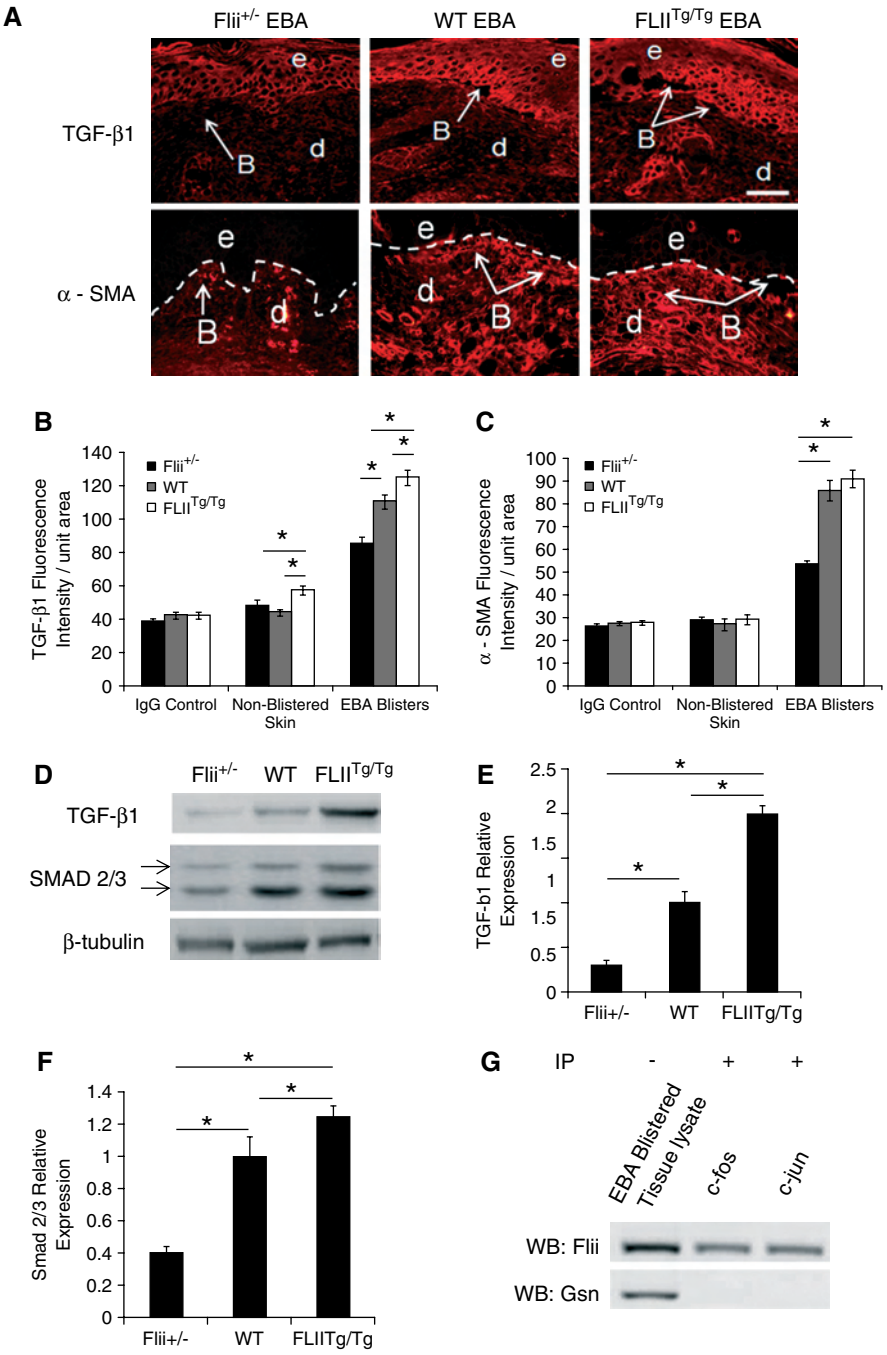

Figure 7. Flii affects TGF-β1/Smad signalling during skin blistering. (A–C) *Flii*$^{+/-}$ EBA mice have significantly decreased TGF-β1 and α-SMA expression in blister skin compared with WT and *FLII*$^{Tg/Tg}$ EBA mice. $n = 4$. Original magnification: ×40. e = epidermis; d = dermis; B = blister; dotted line = dermal–epidermal junction. Scale bar in A = 250 μm. (D–F) Western blot analysis of TGF-β1 signalling members using protein extracted from blistered skin of *Flii*$^{+/-}$, WT, and *FLII*$^{Tg/Tg}$ EBA mice. Reduced Flii levels significantly reduce TGF-β1/Smad signalling, while Flii overexpression leads to up-regulated TGFβ1/Smad signalling. (G) Anti-cFos and c-Jun immunoprecipitates (IP) were prepared from blistered skin of WT EBA mice and immunoblotted for Flii or Gelsolin (positive control) antibodies. Flii co-localizes with AP-1 proteins cFos and cJun. Data are representative of three independent experiments.

incisional and burn wounds [22,30]. Flii is also secreted in response to wounding, suggesting a potential extracellular role for this protein [22].

The present study shows that Flii expression was specifically increased in the blisters of patients of all three main EB types but was most pronounced in the blistered skin of RDEB patients, correlating with the clinical severity of blistering in these patients. In agreement with up-regulated Flii activity in human EB wounds, we also observed increased Flii at the site of blister formation in both EB mouse models investigated. Interestingly, specific differences were observed in the pattern of expression of Flii between human and mouse blistered skin, which will be further investigated in future studies. Flii was increased in EBA blistered skin and in the blisters of ColVII hypomorphic mice, particularly in the front paws, which exhibited TGF-β1-mediated fibrosis and pseudosyndactyly. When mice

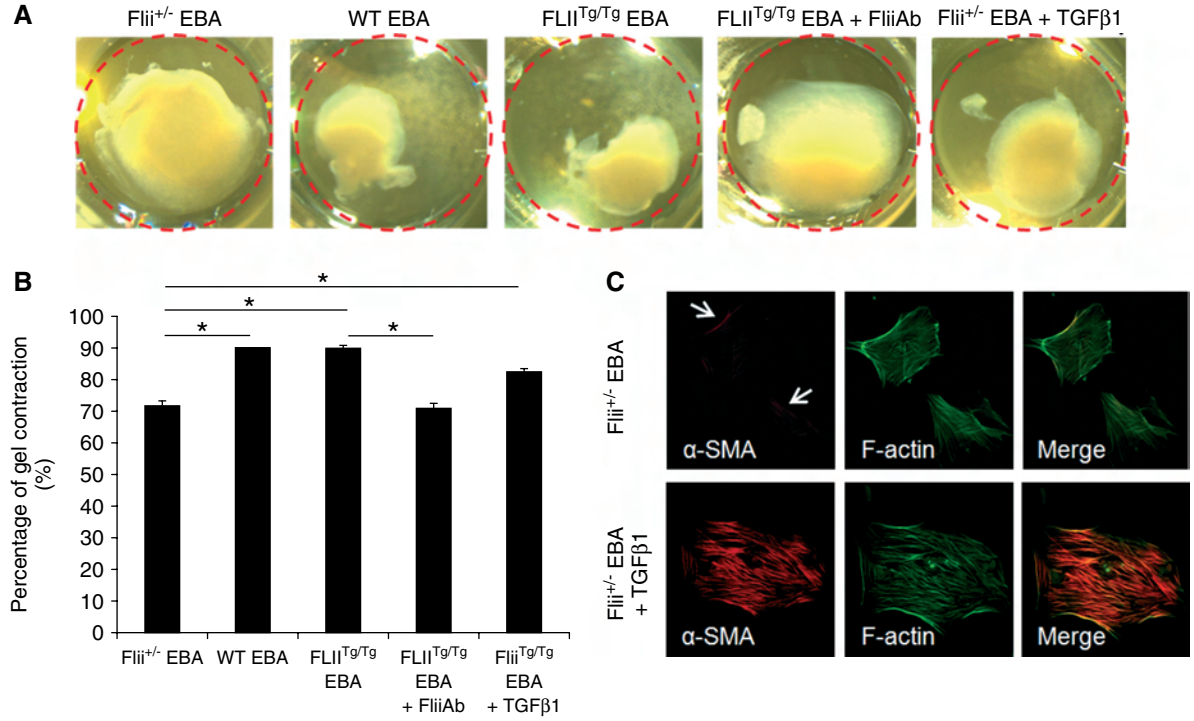

Figure 8. TGF-β1 mediates Flii effects on collagen contraction and fibroblast differentiation. (A, B) Treatment of *FLII*$^{Tg/Tg}$ EBA fibroblasts with Flii neutralizing antibody (FliiAb) significantly reduces collagen gel contraction, comparable to *Flii*$^{+/-}$ EBA mice fibroblasts, while treatment of *Flii*$^{+/-}$ fibroblasts with TGF-β1 increases the collagen gel contraction. $n = 6$. (C) TGF-β1-mediated development of α-SMA-positive stress fibres is Flii-dependent. Arrows show α-SMA in non-treated control *Flii*$^{+/-}$ EBA fibroblasts. The experiment was repeated in triplicates. Mean ± SEM. $^{*}p < 0.05$.

with different levels of *Flii* gene expression were induced to form blisters using the EBA model, a three-fold increase in Flii expression was observed in skin of *FLII*$^{Tg/Tg}$ EBA mice as opposed to a two-fold increase observed in wild-type EBA mice. This increase in Flii expression correlated with increased blister severity. Conversely, reducing *Flii* gene expression by 50% in the heterozygous knockout led to a reduced incidence of blisters with decreased blister severity, indicating that down-regulation of Flii could lead to improved outcomes for EB patients.

Flii influences wound healing through modulation of integrin-mediated cellular adhesion during wound healing [16,39]. Flii interacts with talin, which alters the pool of talin available for the binding and activating of integrin receptors, hence leading to changes in cellular adhesion and migration [16,39]. Integrin chains α3, α6, and β1 were investigated in the blistered skin of *Flii*$^{+/-}$, wild-type, and *FLII*$^{Tg/Tg}$ EBA mice, and found to be significantly elevated. No significant difference was observed in the expression of integrin α6 in mice with differential *Flii* expression; however, compared with both *Flii*$^{+/-}$ and wild-type EBA blisters, *FLII*$^{Tg/Tg}$ EBA blisters had impaired up-regulation of integrin α3 and β1 chains. Different integrin receptor expression and their affinity for extracellular matrix ligands adversely affect cellular migration, adhesion, and fibroblast-mediated wound contraction; therefore, Flii effects on integrin expression in blisters may influence the wound-healing ability of these lesions.

Type VII collagen (ColVII) is the major component of anchoring fibrils, providing stability to the dermal–epidermal junction [40]. TGF-β1 is an important regulator of mesenchymal–epithelial interactions and regulates ColVII deposition through Smad-dependent binding to the *COL7A1* gene promoter and interaction with AP1 transcription factor [41–44]. Indeed, previous results suggest a relationship between Flii and TGF-β responses during wound repair [30]. Changes in Flii expression in response to blister formation may therefore affect ColVII fibrilogenesis. Examination of the unwounded skin of *Flii*$^{+/-}$, wild-type, and *FLII*$^{Tg/Tg}$ mice showed significantly decreased ColVII expression in *Flii*-overexpressing mice compared with both *Flii*$^{+/-}$ and wild-type mice, while *Flii* deficiency improved ColVII expression. These findings, in conjunction with previous studies showing increased numbers of hemidesmosomes in *Flii*$^{+/-}$ mice skin [16], suggest that Flii may modulate dermal–epidermal adhesion. Interestingly, although *FLII*$^{Tg/Tg}$ mice do have thinner, more fragile skin [16], they do not have an overt blistering phenotype. However, only 35% of normal ColVII expression is required for skin to maintain its stability and barrier function [45]. Moreover, a recently developed ColVII hypomorphic mouse model showed that the presence of only 10% of normal ColVII expression confers sufficient stability to the dermal–epidermal junction for long-term survival, albeit with sub-epidermal blistering and the development of symptoms resembling RDEB [32,46].

Keratinocytes and fibroblasts derived from *Flii*$^{+/-}$, wild-type, and *FLII*$^{Tg/Tg}$ EBA-induced mice retain a cell-specific effect of Flii on cellular adhesion, with decreased cellular adhesion in cells extracted from *Flii*-overexpressing mice. Fibroblasts, but not keratinocytes, showed a further decrease in cell adhesion in response to induction of EBA, suggesting that they may play a significant role in EBA. Indeed, recent studies have identified fibroblasts as a significant source of ColVII anchoring fibrils which contribute to skin stability *in vivo* [45]. Studies have also demonstrated the therapeutic potential of fibroblasts in EB [32,47–49]. Reducing *Flii* gene or protein expression rescued the EBA-induced fibroblast cell phenotype and improved cellular adhesion and proliferation, suggesting that the presence of Flii in fibroblasts is a negative contributing factor in EBA.

TGF-β1 is a key growth factor involved in scarring in RDEB [32,43,44,50]. TGF-β binds to the *COL7A1* promoter, and therefore modulators of TGF-β may be valuable in the prevention of excessive fibrosis in EB patients [44]. Flii affects TGF-β gene expression in response to burn injury, and reducing *Flii* gene or protein expression using a neutralizing antibody against Flii decreases TGF-β1 expression [30]. In this study, blisters of *Flii*$^{+/-}$ EBA mice had decreased levels of pro-fibrotic TGF-β1 and reduced numbers of α-SMA-positive myofibroblasts compared with both wild-type and *FLII*$^{Tg/Tg}$ EBA mice lesions, suggesting that Flii may potentially modulate blister severity via affecting TGF-β1. Flii associated with AP-1 proteins cFos and cJun in blistered skin, and reducing *Flii* expression decreased TGF-β1/Smad signalling. Conversely, increasing *Flii* expression up-regulated TGF-β1/Smad signalling. Flii has previously been identified as a nuclear receptor co-activator [51]; therefore, it is possible that Flii may be able to form a transcription complex with AP-1 proteins and thereby affect TGF-β1 signalling. Examination of the functional effect of Flii on collagen contraction showed that *Flii*$^{+/-}$ fibroblasts from EBA-induced mice, with decreased TGF-β1 levels, had reduced contractile ability which in a fibrotic condition would be expected to result in reduced contracture. *Flii*$^{+/-}$ fibroblasts from EBA-induced mice showed a reduced assembly of α-SMA-positive stress fibres, and addition of exogenous TGF-β1 enhanced the contractile activity with an increased assembly of α-SMA-positive stress fibres and collagen gel contraction *in vitro*. Similarly, *FLII*$^{Tg/Tg}$ fibroblasts extracted from EBA-induced mice and treated with a FliiAb were less able to contract collagen. While collagen contraction is an important prerequisite of a healing wound, excessive contraction and fibrosis contribute to hypertrophic scarring and pseudosyndactyly, both of which are major problems for RDEB sufferers and in EBA. These results demonstrate the therapeutic potential of FliiAb for treatment of EBA and a functional significance of decreased *Flii* gene expression in skin blistering.

In summary, Flii is elevated in the blisters of EB patients, and up-regulation of Flii increases the severity of blistering in mouse models of RDEB and EBA. Reducing Flii expression lowers the incidence and severity of the blisters, improves cellular responses, and reduces collagen contraction. Flii affects TGF-β1 and Smad 2/3 expression, and the exogenous addition of TGF-β1 to *Flii*$^{+/-}$ fibroblasts from EBA-induced mice restores the contractile phenotype of these cells, suggesting that an interplay between Flii and TGF-β1 may underscore the function of Flii in EB. Therefore, therapies aimed at reducing Flii within the blisters of patients with EB may be a novel approach for reduced blistering and improved wound healing in these patients.

Acknowledgment

This work was supported by grants from the National Health and Medical Research Council of Australia (No 626802 to AJC, RMA, and DFM) and Channel 7 Children's Research Foundation (to AJC, RMA, and DFM), as well as grants from Deutsche Forschungsgemeinschaft (Nos EXC 306/1 and LU 877-1 to DZ and RJL). AJC is supported by a National Health and Medical Research Council of Australia Senior Research Fellowship. We wish to acknowledge Drs Wei Melbourne, Wenfei Yan, and Kim Tran at the Departments of Dermatology and Pathology, St George Hospital, Sydney for their assistance in the provision of human samples; the research team at the Department of Dermatology, University Medical Center Freiburg, Freiburg for breeding of the ColVII hypomorphic mice and technical assistance; and the Dystrophic Epidermolysis Bullosa Research Association of South Australia for their support.

Author contribution statement

AJC, RMA, and ZK conceived the experiments. ZK carried our all experiments and analysis with the assistance of XLS. DZ, RJL, and MH provided ColVII antibody and initially developed the EBA mouse model. LBT and JSK bred the ColVII hypomorphic mice. DFM collected and helped to analyse all human samples. ZK and AJC wrote the manuscript, and all authors contributed to the manuscript preparation and approved the final submitted and published versions.

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Note: Reference 52 is cited in the Supporting information to this article.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Supplementary materials and methods

Figure S1. Modulation of *Flii* levels affects the adhesion of fibroblasts from EBA-induced mice.