Topically Applied Flightless I Neutralizing Antibodies Improve Healing of Blistered Skin in a Murine Model of Epidermolysis Bullosa Acquisita

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Epidermolysis bullosa (EB) is a chronic inheritable disease that leads to severe blistering and fibrosis. Previous studies have shown that the actin cytoskeletal protein flightless I (Flii) impairs wound healing associated with EB. Using a mouse model of EB acquisita (EBA), the effect of "mopping up" Flii using Flii-neutralizing antibodies (FnAbs) before, during, and after blister formation was determined. FnAbs, incorporated into a cream vehicle and applied topically to the skin, penetrated into the basal epidermis and upper papillary dermis but were not detected in serum or other organs and did not alter neutrophil or macrophage infiltration into the blistered skin. Histological assessment of blister severity showed that treatment of early-stage blisters with FnAb cream reduced their severity and improved their rate of healing. Treatment of established blisters with FnAb cream also improved healing and restored the skin's tensile strength toward that of normal skin. Repeated application of FnAbs to EBA skin before the onset of blistering reduced the severity of skin blistering. Independent of when the FnAbs were applied, skin barrier function and wound healing were improved and skin fragility was reduced, suggesting that FnAbs could potentially improve healing of patients with EB.

INTRODUCTION

Inheritable epidermolysis bullosa (EB) comprises a group of 30 phenotypically or genotypically distinct disorders characterized by recurrent blistering as a result of structural fragility of skin and mucous membranes (Uitto, 2009). Chronic wound healing associated with trauma-induced blistering has a severely negative effect on the patient's quality of life. Impaired healing of blisters often results in infections, scarring, and development of aggressive squamous cell carcinoma, which lead to metastasis and mortality early in life (Bruckner-Tuderman, 2010). Mutations of at least 14 genes encoding for proteins responsible for the maintenance of the keratinocyte structural stability or adhesion of keratinocytes to the underlying dermis give rise to differences in the site of ultrastructural cleavage between the main EB types. EB simplex is characterized by intraepidermal blistering; junctional EB and dystrophic EB are characterized by the blistering within the lamina lucida and sub-lamina densa, respectively, whereas Kindler syndrome can present with multiple cleavage plains (Fine, 2010). Patients can also develop an acquired form of EB called EB acquisita (EBA), where autoimmunity against type VII collagen results in subepidermal blistering and clinical features similar to those observed in dystrophic EB patients (Ishii et al., 2010).

Recent advances in a number of novel molecular strategies including ex vivo gene therapy (Mavilio et al., 2006; De Luca et al., 2009), stem cell transplantation (Tolar et al., 2009; Tamai et al., 2011), and administration of allogeneic fibroblasts (Wong et al., 2008; Nagy et al., 2011) or recombinant protein (Fritsch et al., 2009; Remington et al., 2009) have offered hope for the development of therapies for patients with EB; however, many technical and safety problems are still encountered (Uitto et al., 2010, 2011). In addition, EBA is highly resistant to immunosuppressive agents, making current treatment difficult (Ishii et al., 2010). This identifies the need for safer and effective alternatives for the treatment modalities of all EB types.

The actin-remodeling protein flightless I (Flii) has an important role in mediating cellular adhesion, hemidesmo-some structure, and collagen deposition (Kopecki et al., 2009,

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Abbreviations: EB, epidermolysis bullosa; EBA, epidermolysis bullosa acquisita; Flii, flightless I; FnAb, flightless I–neutralizing antibody; TEWL, transepidermal water loss

2011a), with key roles in development, wound healing, and tissue regeneration (Campbell et al., 2002; Lin et al., 2011; Waters et al., 2011). Flii L-rich repeat domain also allows for interaction with numerous proteins (Fong and de Couet, 1999; Kopecki et al., 2009, 2011b) and regulation of cellular functions, including migration and proliferation (Cowin et al., 2007), transcriptional regulation (Archer et al., 2004), focal adhesion turnover (Kopecki et al., 2011b), toll-like receptor signaling (Dai et al., 2009), inflammation, cytokine production (Li et al., 2008), and cell division (Deng et al., 2007). Although the loss of Flii gene leads to embryonic lethality in both Drosophila (Campbell et al., 1993) and the mouse (Campbell et al., 2002), the overexpression of Flii gene leads to thinner more fragile skin, impaired wound healing, and increased scarring (Cowin et al., 2007; Kopecki et al., 2009).

Previous studies have demonstrated the negative role for Flii in wound healing leading to interrupted skin homeostasis, skin fragility, diffuse arrangements of ColVII-anchoring fibrils, and impaired cellular adhesion, migration, and proliferation (Cowin et al., 2007; Kopecki et al., 2009, 2011b). Having a unique structure to other members of the gelsolin family of actin-remodeling proteins, Flii has the ability to transduce the cell signaling events into cytoskeleton remodeling and therefore link different signaling pathways with the actin cytoskeleton. Flii is increased in blisters of EB patients, and its upregulation correlates with the severity of blistering seen in mouse models of recessive dystrophic epidermolysis bullosa and EBA (Kopecki et al., 2011a). We have also described the increased dermal–epidermal separation and altered TGF-β1/Smad signaling and collagen-mediated contraction and fibrosis associated with increased levels of Flii gene (Kopecki et al., 2011a). Neutralizing the extracellular Flii activity via intradermal injection of monoclonal Flii-neutralizing antibodies (FnAbs) accelerates wound reepithelialization and improves the macroscopic appearance of early scars in porcine models of wound healing (Jackson et al., 2012). Here, we investigate the effect of topical FnAb cream on the healing of blisters in an autoimmune ColVII murine model of EBA.

RESULTS
Topical application of FnAb cream modulates Flii expression in the skin
Flii is present in the blistered skin of human patients and mice models of recessive dystrophic epidermolysis bullosa and EBA. To determine whether it is also secreted, a suction model of skin blistering was used. Flii levels were analyzed in human blister fluid and was found to be present in blister wound fluid and for the two consecutive days post blister induction (Figure 1a). Compared with acute wound fluid control, the level of Flii was significantly elevated in human blister fluid taken at the time of blister creation, with reduced levels seen at day 1 and day 2 post blistering (Figure 1a and b). We have previously injected FnAbs (50 μg ml^{-1}) intradermally to murine and porcine wounds (Cowin et al., 2007; Adams et al., 2009; Jackson et al., 2012). To determine whether FnAbs could be delivered directly to skin and blisters in a

topical cream preparation, FnAb cream was prepared as described in the Materials and methods by mixing aqueous cetomacrogol cream with rabbit polyclonal FnAb (50 μg ml^{-1}) and directly applied to the skin. In addition, FnAb was directly conjugated to DyLight Fluor 488 (50 μg ml^{-1}), and this was also incorporated into aqueous cetomacrogol cream to confirm the penetration of FnAbs into skin. Both the directly conjugated FnAb-DyLight and FnAb alone showed similar penetration into the skin, with localization observed in the basal epidermis and the upper papillary dermis (Figure 1c–h). Addition of FnAb to the surface of the skin leads to decreased expression of Flii in the epidermis (Figure 1e) compared with control IgG cream (Figure 1f). FnAb, detected using indirect immunohistochemistry, colocalized with Flii protein and was found in the epidermis, at the epidermal–dermal junction, and penetrated into the upper papillary dermis with evident intracellular colocalization staining of fibroblasts (Figure 1g and h). The effect of FnAb cream on Flii expression in the skin was further examined in wild-type mice using western blotting. A single application of FnAb cream (50 μg ml^{-1} FnAb) rubbed into the dorsal skin of wild-type mice resulted in reduced expression of Flii (∼60% reduction) for up to 1 day post treatment (Figure 1i–j). At 2 days post treatment, Flii levels had returned to baseline (Figure 1j).

Topical application of FnAb cream is localized to the skin
Flii levels were investigated in different organs of wild-type mice and were compared with the levels of Flii in skin using western blotting and band densitometry. Flii was detected in the heart and lymph nodes at levels lower than those observed in skin; however, levels of Flii in the lung, kidney, and liver were higher than those observed in the skin (Figure 2a and b). An ELISA for detection of FnAbs was developed to test whether the application of FnAb cream to the skin would have a systemic effect. A single topical application of FnAb cream (containing 50 μg ml^{-1} FnAb) or dose-matched IgG control cream was rubbed into the skin of wild-type mice and to the blistered skin of EBA mice. FnAb levels were detected in the skin of wild-type mice up to 3 days post treatment (Figure 2c). FnAb levels were comparable to those observed in control blistered skin (Figure 2c), but were not detected in other organs systemically including heart, lung, kidney, spleen, lymph node, gut, or serum (Figure 2c).

### Treatment of early-stage blisters with FnAb cream reduces their severity in EBA mice
In the immune-mediated model of EB, administration of anti-ColVII antibodies leads to the disruption of ColVII-anchoring fibrils, and skin blistering occurs on the backs of the mice from day 8 post administration. FnAb cream (50 μg ml^{-1} FnAb) was gently rubbed onto the skin of the EBA mice for three consecutive days at the start of blister formation (days 8–10). EBA mice treated with FnAb cream had significantly fewer blisters and reduced number of lesions compared with EBA mice treated with control IgG antibody in a cream vehicle (Figure 3a–c). To determine the effect of FnAb cream on restoration of skin barrier function following skin blistering, the transepidermal water loss (TEWL) was measured in EBA
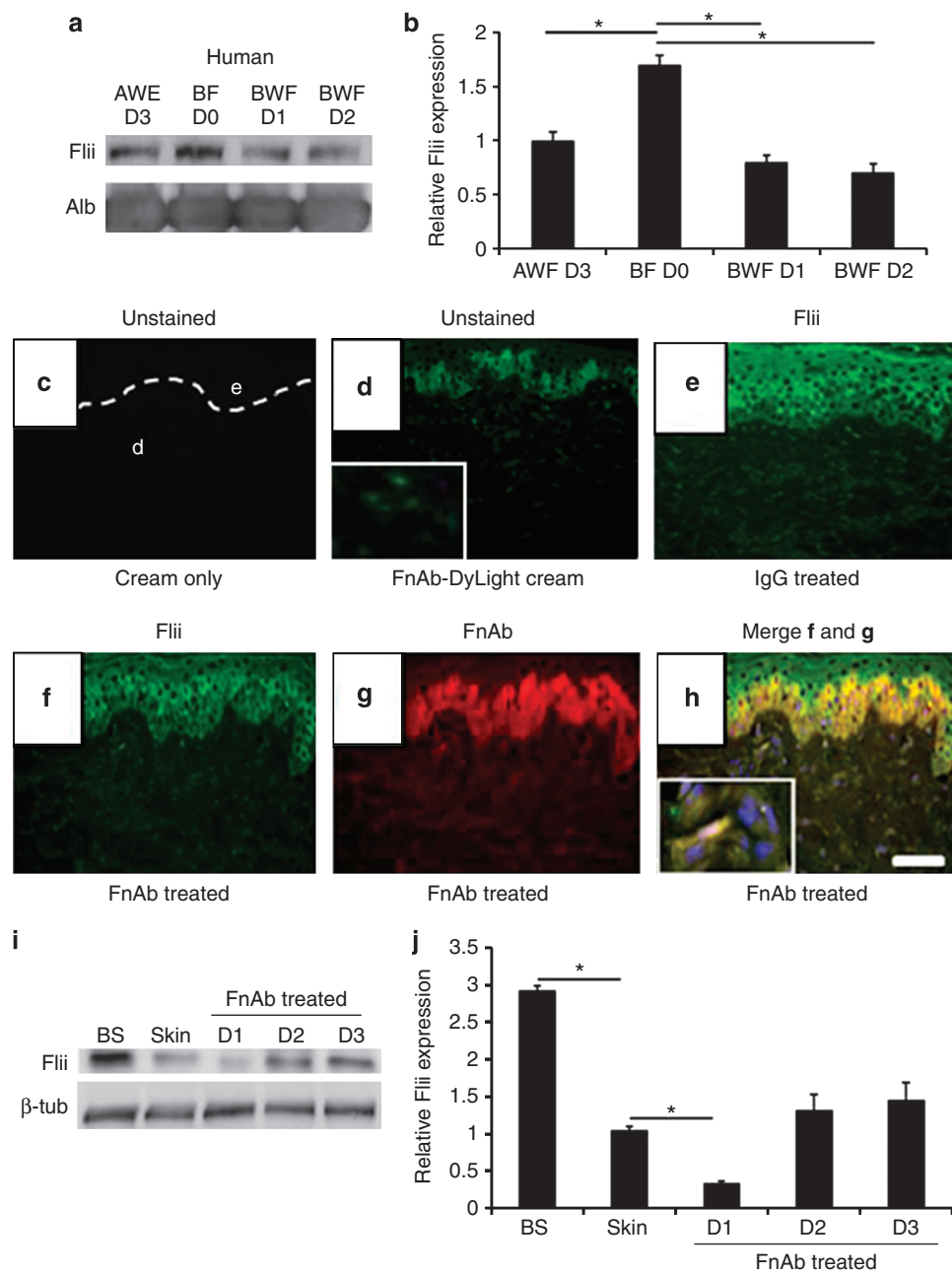
Figure 1. **Flightless I (Flii) is increased in response to blistering and can be modulated using topical Flii-neutralizing antibody (FnAb) cream.** (**a, b**) Flii levels in blister fluid (BF), acute wound fluid (AWF), and blister would fluid (BWF) collected from blisters (days 1–2 post induction) were analyzed using western blotting and band densitometry. (**c–h**) Topical application of cream only, FnAb-DyLight cream, FnAb cream, or IgG control cream on intact porcine skin showing FnAb-Dylight (green in **d**), Flii (green in **e** and **f**), and FnAb (red) localization and association (yellow). 4'6-Diamidino-2-phenylindole (blue). Original magnification, ×20. Insert, ×100. Scale bar = 100 μm. $n = 3$. e, epidermis; d, dermis; dotted line, dermal–epidermal junction. (**i** and **j**) Flii expression in blistered skin (BS)-positive control, nontreated intact skin (skin), or intact skin of wild-type mice following a single FnAb cream application (D1–D3, days post treatment) was analyzed using western blotting and band densitometry. BS, blistered skin; D, day. Albumin (Alb) and β-tubulin (β-tub) staining in **a** and **g** show equal loading. The figure is representative of two independent experiments. Mean ± SEM, *$P > 0.05$.

mice treated with FnAb cream or IgG control cream from day 7 of the experiment. TEWL readings peaked on day 8 in all mice when the blisters started developing, fluctuated following repeated blistering, and returned to basal levels by day 16 of the experiment (Figure 3d). EBA mice treated with FnAb cream had significantly decreased TEWL compared with EBA mice treated with IgG control cream, suggesting an improvement in restoration of the epidermal/dermal barrier of the wounds (Figure 3d). Histological examination of the blister lesions of EBA mice treated with FnAb compared with IgG control revealed a thinner epidermis similar to intact skin. There was also decreased influx of polymorphic cells,
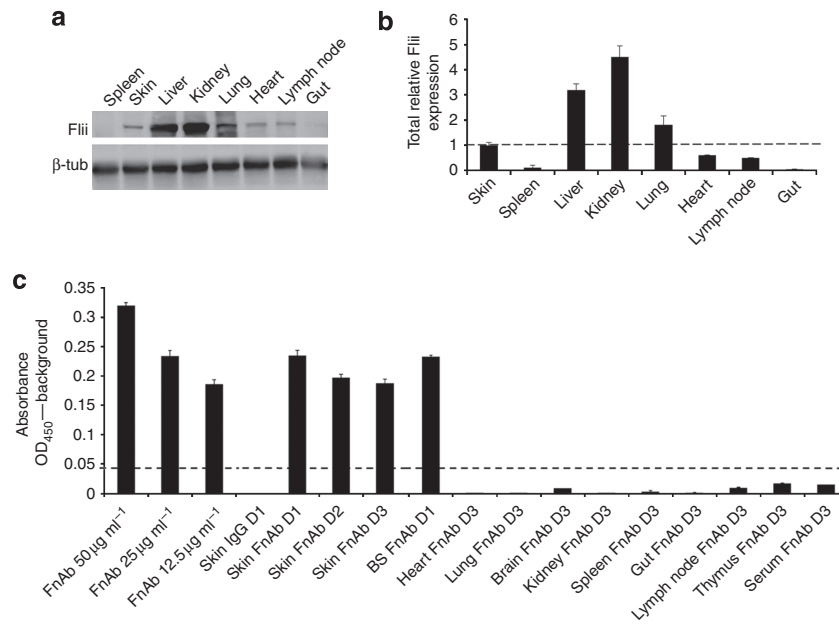
Figure 2. Topical application of flightless I (Flii)-neutralizing antibody (FnAb) cream is localized to the skin. (**a**, **b**) Flii expression in different organs of wild-type mice was assessed using western blotting and band densitometry. Expression was normalized to β-tubulin (β-tub) levels. Dotted line, expression of Flii in intact skin. (**c**) Following a single topical application of FnAb cream or IgG control cream to intact skin, levels of FnAb were detected in intact skin (up to 3 days following treatment) but not in other organs or serum. BS, blistered skin; D, day. FnAb was not detected in IgG-treated skin (negative control) but was detected in FnAb-blistered skin (positive control). Figure is representative of two independent experiments. Dotted line, positive signal. Mean ± SEM.

significantly decreased histological blister scores, and improved rate of healing (Figure 3e and f).

Treatment of mature blisters with FnAb cream improves healing and restores the skin's tensile strength

The timing of the application of the FnAb cream was investigated by applying FnAb cream for three consecutive days when the blisters were at their peak severity (day 12–14). EBA mice treated with FnAb cream had lower numbers of lesions and a lower percentage of skin blistering. In contrast, EBA mice treated with IgG control cream had large numbers of lesions and scabs covering existing blisters (Figure 4a and b). The blisters of these mice treated with FnAb cream at days 12–14 also had a faster restitution of the barrier function and significantly decreased histological blister scores compared with skin blistering observed in EBA mice treated with IgG control cream (Figure 4c–e). To examine the functional effect of FnAb on intact or blistered skin, skin strips from wild-type mice and EBA mice treated with FnAb cream or IgG control cream were assessed for their tensile skin strength. Application of FnAb cream to intact skin of wild-type mice resulted in an increased trend toward stronger skin; however, this was not statistically significant. Induction of EB significantly reduced the tensile strength of the skin; however, treatment with FnAb cream led to significantly stronger, less fragile skin (Figure 4f).

Repeated application of FnAb cream to skin during induction of EBA before the onset of blistering reduces the extent of blistering

To determine whether FnAb cream could delay the onset of skin blistering in an EBA mouse model, mouse skin was treated with FnAb cream on alternate days over the first 10-day period during induction of EBA. Both EBA mice groups treated with FnAb cream or IgG control cream developed skin blistering; however, those treated with FnAb cream had significantly fewer lesions, and the extent of skin blistering was decreased at days 8–12 (Figure 5a–c). The FnAb cream–treated EBA mice had decreased disruption of the skin barrier function with lower TEWL values and significantly decreased histological blister scores (Figure 5d and e). Repeated application of FnAb cream to skin restored skin tensile strength with significantly stronger blistered skin of EBA FnAb cream–treated mice compared with blistered skin of EBA mice treated with IgG control cream (Figure 5f).

Effect of FnAbs on dermal immunological responses of EBA mice

Application of xenogeneic antibodies, particularly when applied in repeated doses, can induce changes in inflammatory responses. To determine whether changes in immunological reactions were produced in response to repeated administration of FnAbs, the cellular infiltration of neutrophils and macrophages was assessed. Blistered skin of mice treated with FnAb or IgG control was assessed at day 12 or day 16. Inflammatory cell infiltrate in blistered skin was reduced at day 16 of the experiment compared with day 12; however, no statistically significant difference was observed in FnAb- or IgG-treated skin in either neutrophil or macrophage cell numbers (Supplementary Figure S1 online).

DISCUSSION

Studies have shown that modulation of Flii activity by either genetic knockdown or intradermal injection of FnAbs in both
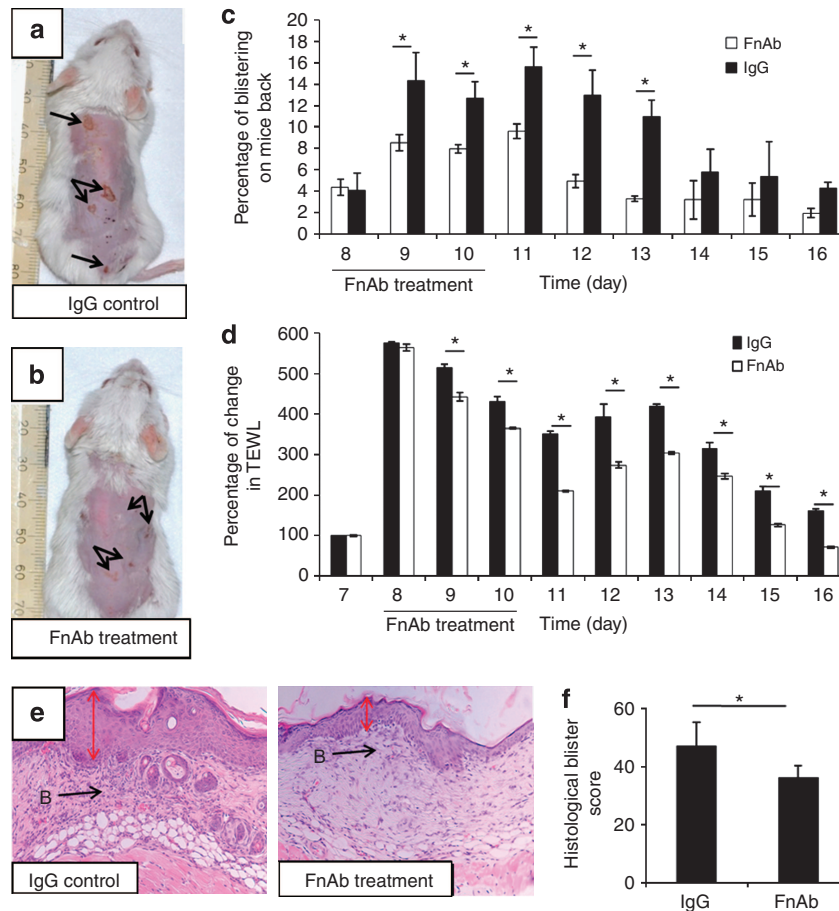
Figure 3. Topical application of flightless I-neutralizing antibody (FnAb) cream during early blister development significantly reduced the severity of blistering in epidermolysis bullosa acquisita (EBA) mice (a–c) Images of EBA mice (day 12) treated with FnAb cream or IgG control cream over 3 consecutive days (8–10) during early blister development were analyzed for macroscopic blister wound area using Image Pro-Plus. (d) Graphical representation of transepidermal water loss (TEWL) in EBA mice treated with FnAb cream or IgG control cream. (e, f) Representative images of hematoxylin and eosin–stained blister wounds at day 16 of the experiment, and graphical analysis of histological blister score in EBA mice, treated with FnAb cream or IgG control cream. B, blister wound. Red arrow, epithelium thickness. $n = 4$. Mean ± SEM, $*P > 0.05$.

small and large animal models of wound healing and burn injury improve the rate of healing and reduce early scar formation (Cowin *et al.*, 2007; Adams *et al.*, 2009; Jackson *et al.*, 2012). Flii is also secreted in response to wounding, suggesting a potential extracellular role for this actin-remodeling protein (Cowin *et al.*, 2007). To our knowledge previously unreported, treatment of blistered skin with FnAb cream reduces the severity and extent of blister formation in a mouse model of EBA. FnAbs applied to intact or blistered skin in a cream vehicle are absorbed into the epidermis and papillary dermis, and can be detected in the skin for up to 3 days and can effectively reduce endogenous Flii protein expression. The systemic effect of FnAb applied to the skin was investigated, and while FnAbs were detected in the skin locally for up to 3 days post application, they were not found in serum or other organs, suggesting that the application of FnAb cream to the skin remains localized to the skin.

Increased Flii levels contribute to impaired wound healing, skin fragility, and increased dermal–epidermal separation (Cowin *et al.*, 2007; Kopecki *et al.*, 2009, 2011a). The effect

of FnAb cream on the healing of blisters was investigated using a murine model of EBA. In this model, mice develop widespread blistering on their backs from day 8 of the experiment, peaking at day 12, with healing occurring by day 16 (Sitaru *et al.*, 2005; Kopecki *et al.*, 2011a). Initially, mice backs were treated with topical FnAb cream during the development of the blisters (day 8–10), with up to 50% reduction in the extent of skin blistering being observed and decrease in TEWL, suggesting an improvement in the protective skin barrier function. In agreement with previous studies describing the positive effect of reduced Flii expression (genetically or using FnAb) on cellular migration, proliferation, and adhesion, as well as improved wound healing (Cowin *et al.*, 2007; Kopecki *et al.*, 2009), EBA mice treated with FnAb cream had a significantly decreased number of blister lesions and lower histological blister scores. These findings are in agreement with improved wound reepithelialization and decreased blistering observed in $Flii^{+/-}$ EBA mice (Kopecki *et al.*, 2011a) and improved healing observed in response to intradermal injection of FnAb into porcine excisional wounds
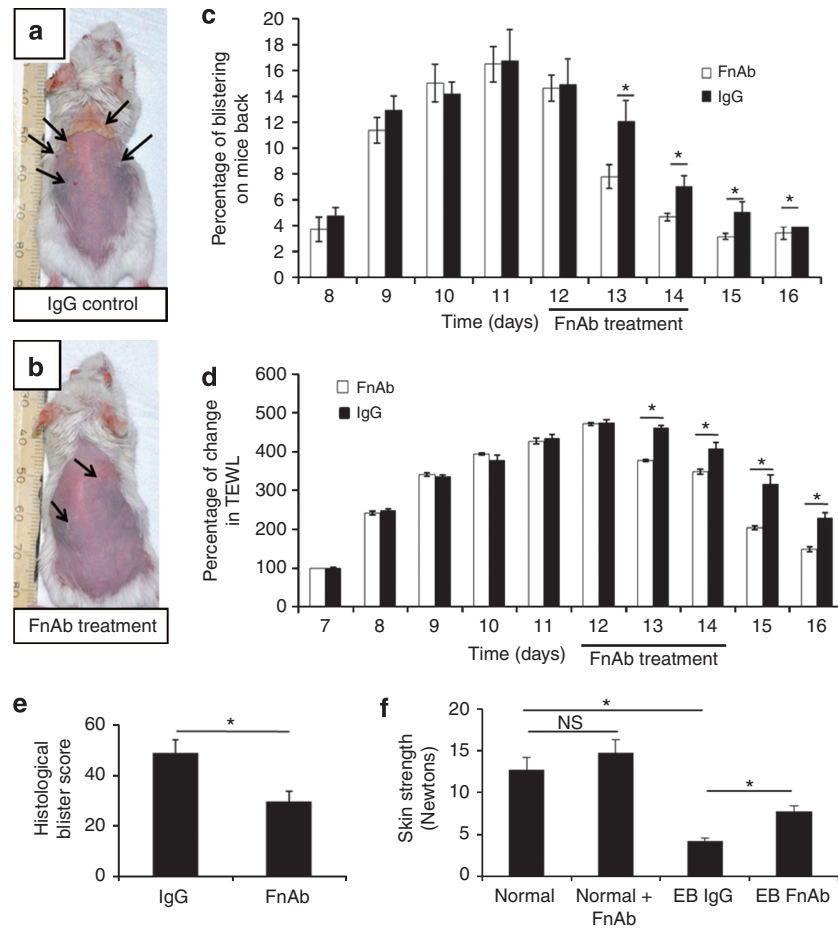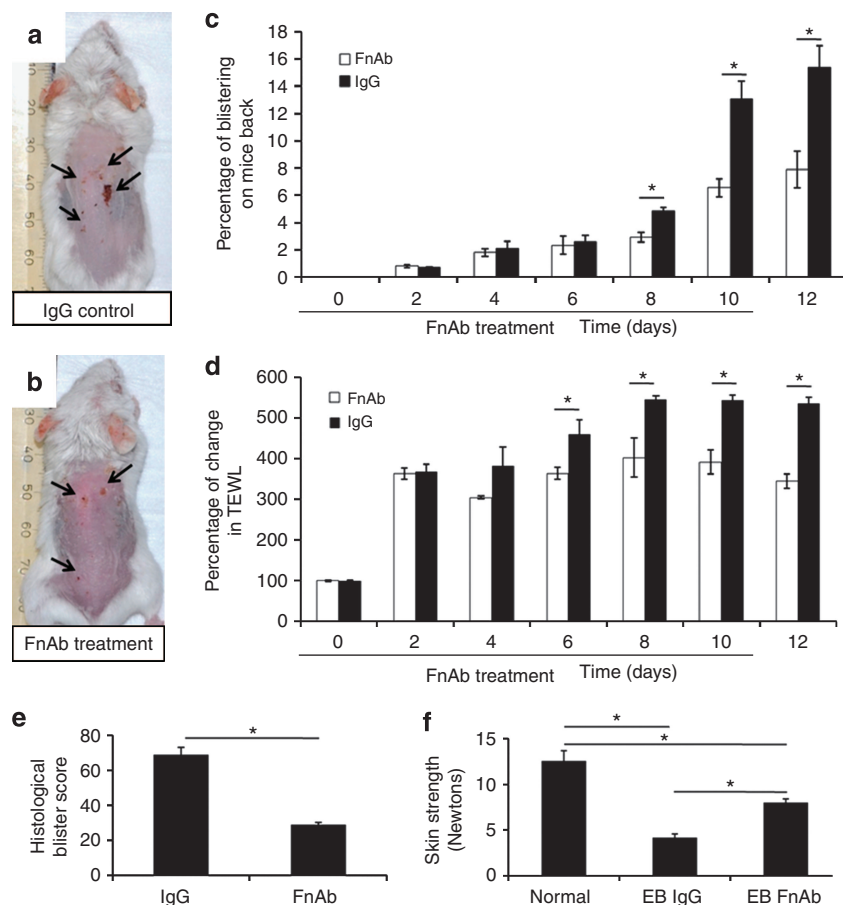
Figure 4. Flightless I-neutralizing antibody (FnAb) cream treatment of mature blisters improves healing and increases skin tensile strength. (a–c) Images of epidermolysis bullosa acquisita (EBA) mice blisters (day 15) treated with FnAb cream or IgG control cream over three consecutive days (12–14) were analyzed for macroscopic blister wound area using Image Pro-Plus. (d) Graphical representation of transepidermal water loss (TEWL) in mice treated with FnAb or IgG control. (e) Graphical representation of the histological blister score indicative of the rate of blister healing following treatment of developed blisters with FnAb cream or IgG control cream. (f) Graphical representation of skin tensile strength in normal intact skin, following treatment with FnAb cream, and in mature blisters, following treatment with FnAb cream or IgG control cream. $n = 4$. Mean ± SEM, *$P > 0.05$. NS, not significant.

(Jackson *et al.*, 2012). Indeed, wounds treated with FnAb show an increased rate of reepithelialization and granulation tissue formation associated with improved wound healing and macroscopic appearance of early scars (Jackson *et al.*, 2012). Improved wound reepithelialization is vital for the restoration of the skin barrier function.

Formation of a strong skin barrier contributes to skin strength, whereas the quick recovery or reestablishment of the skin barrier post blistering is critical for healing of blisters. Application of FnAb cream to intact skin before blister development (day 0–10) or to mature and healing blisters (day 12–14) also decreased the severity and incidence of skin blistering (up to 50%), with improved restoration of the protective skin barrier. Interestingly, application of FnAb cream to intact skin of wild-type mice resulted in an increasing trend toward stronger skin and while this was not significant it suggesting that reducing Flii levels when Flii is not upregulated may also be beneficial for increasing skin tensile strength. Of particular interest was the finding that the application of FnAb cream significantly improved the skin

barrier function and reduced the skin fragility (up to 50%), suggesting it might be able to reduce the fragility of EB skin and help decrease the severity of blister formation observed in these patients.

The uptake of oil-soluble agents is facilitated by the oil-permeable nature of the skin. Previous studies using topical administration of antibodies formulated in water-in-oil microemulsion have shown the possibility of reducing inflammation in mice skin (Himes *et al.*, 2010). Despite our repeated topical administration of a xenogeneic antibody to the skin, no differences in neutrophil or macrophage cell infiltration were observed. In addition, FnAbs were not detected in the serum or in distal organs of the treated mice, suggesting that FnAbs could be a potential treatment modality to be considered for further development. Additional studies are still required to investigate the toxicology, pharmacokinetics, and effectiveness of FnAb cream before the commencement of any human clinical trials with EB patients; however, the results presented here suggest that FnAbs could potentially be used for the treatment of blisters in EB. In addition, FnAb treatment could

Figure 5. Repeated application of flightless I-neutralizing antibody (FnAb) cream as a preventative measure reduces the severity of blister formation in epidermolysis bullosa acquisita (EBA) mice. (**a**–**c**) Images of EBA mice blisters (day 12) treated with FnAb cream or IgG control cream over 10 alternate days were analyzed for macroscopic blister wound area using Image Pro-Plus. (**d**) Graphical representation of transepidermal water loss (TEWL) in mice treated with FnAb cream or IgG control cream. (**e**) Graphical representation of the histological blister score indicative of the rate of blister healing following preventative application of topical FnAb cream or IgG control cream to mice skin. (**f**) Graphical representation of skin tensile strength following preventative application of topical FnAb cream or IgG control cream to mice skin. $n = 4$. Mean ± SEM, $*P > 0.05$.

be used as a complementary approach to other treatment modalities currently under investigation, including protein and cell replacement therapies. Advantages of the development of topical therapies for improved healing of blistered skin in patients with EB include simple non-painful application during dressing changes, with no viruses or living cells involved in the delivery and local response to treatment. In conclusion, Flii expression can be reduced using a repeated application of FnAb cream, resulting in decreased blister severity, reduced skin fragility, and improved skin barrier function.

MATERIALS AND METHODS
Antibodies
Mouse monoclonal anti-Flii antibody (sc-21716), rat monoclonal neutrophil marker anti-NIMP-R14 (sc-59338), goat polyclonal macrophage marker anti-F4/80 (A-19) (sc-26642), and mouse monoclonal anti-Albumin antibody (sc-51505) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Affinity-purified rabbit anti-flightless (FnAb) raised against the L-rich repeat domain of flightless protein was made by Mimotopes Pty (Clayton, Victoria, Australia) as previously described (Adams *et al.*, 2009). Murine IgG irrelevant antibody (I8765), β-tubulin antibody, and 4′6-diamidino-2-phenylindole

nucleic acid stain were obtained from Sigma (St Louis, MO). Alexa-Fluor 488 goat anti-rabbit (A11008) and Alexa-Fluor 594 goat anti-mouse (A11020) antibodies were obtained from Invitrogen Australia Pty (Waverley, New South Wales, Australia).

Human samples
The clinical investigations were conducted under approval from SA Department of Health Human Research Ethics Committee, in accordance with the Declaration of Helsinki principles and with written informed consent. Suction blisters were induced on the upper arms of six healthy volunteers, with no known dermatological condition as previously described (Koivukangas and Oikarinen, 2003). Briefly, blisters were induced using a blister suction device (GlaxoSmithKline, Melbourne, Victoria, Australia) tightened onto the forearm and connected to the pump for 1 hour under a constant pressure of 90 kPa. The suction results in the separation of the epidermis from the dermis at the level of lamina lucida in the basement membrane, resulting in blister cavity filled with tissue fluid consisting of interstitial fluid and small portion of proteins from the serum. Blisters were lanced and fluid was collected using an insulin syringe. Blister wounds were dressed daily using Tegaderm transparent film dressings (3M, St Paul, MN), and blister wound fluid was collected from the dressings at day

1 and day 2 post initial blister induction. Acute wound fluid control was collected from patients attending the Wound Clinic, The Queen Elisabeth Hospital, Adelaide. Protein from wound fluid samples was precipitated using standard trichloroacetic acid methods as previously described (Jiang *et al.*, 2004), and western blotting was performed following standard methods previously described (Kopecki *et al.*, 2009).

FnAb cream formulation

FnAb cream formulation was prepared by mixing the aqueous hypoallergenic Sorbolene cream (cetomacrogel cream without glycerol) with rabbit polyclonal FnAb ($50\,\mu g\,ml^{-1}$). The Sorbolene cream used in these experiments is the aqueous cream that is well absorbed into the skin, contains no active ingredients, and is routinely used for preparations of various dermatological treatments. The concentration of FnAb used in these studies is same as the effective dose determined in our previous *in vivo* studies. The FnAb cream ($200\,\mu l$) was gently rubbed into the skin of mice, and volumes of FnAb cream formulation used were enough to allow the majority of the cream to be absorbed by the skin by the time the mice recovered from anesthesia. No dressings were applied in this study. FnAb-DyLight cream used in porcine *ex vivo* experiments was prepared in above described method and concentrations, except FnAb antibody was firstly conjugated to DyLight Fluor 488 following manufacturer's instructions, DyLight Fluor 488 Antibody Labeling Kit (53024) (Thermo Scientific, Rockford, IL).

Animal studies

Intact skin from male Large White pigs was collected for *ex vivo* experiments and immunohistochemistry following approval from the University of Adelaide Ethics Committee and the IMVS Ethics Committee following the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Briefly, intact porcine skin was mounted on a sterile disk and pinned out using four sterile 25-gauge needles, taking care to preserve the natural tension of the skin. Six explants ($n=3$ pigs) were cultured in DMEM supplemented with 10% FBS at the air–liquid interface, with the epidermis being exposed to air and the dermis being submerged in medium. Hundred microliter of cream only, FnAb-DyLight Fluor 488 cream ($50\,\mu g\,ml^{-1}$ fluorescence-labeled FnAb-DyLight in aqueous cetomacrogol cream), FnAb cream ($50\,\mu g\,ml^{-1}$ FnAb in aqueous cetomacrogol cream), or dose-matched IgG control cream was rubbed into the epidermis and the explants were returned to the $37\,^{\circ}C$ $5\%CO_2$ incubator and incubated for 3 days. After this period, skin explants were collected, formalin fixed, and paraffin embedded. Sections of the skin were processed for immunohistochemical detection of Flii and FnAb.

All studies performed in mice were with wild-type mice of BALB/c background. All experiments were approved by the Animal Ethics Committees of the Adelaide Child, Youth, and Women's Health Service following the Australian Code of Practice for the Care and the Use of Animals for Scientific Purposes. EBA was induced in 3–4-week-old wild-type mice with rabbit anti-mouse ColVII antibody injected subcutaneously as described previously (Kopecki *et al.*, 2011a). To facilitate easier identification of blisters and application of FnAb cream, at the start of the experiments, mice back skin was shaved and hair was removed using hair removal cream (Veet, Reckitt Benckiser, West Ryde, New South Wales, Australia), followed by washing with PBS to ensure no residual hair removal cream was interfering with experiments. Mice back skin was treated with $200\,\mu l$ of FnAb cream ($50\,\mu g\,ml^{-1}$ FnAb in aqueous cetomacrogol cream) by gentle rubbing of the cream onto the skin ($n=4$/time point). A dose-matched irrelevant IgG control cream was also used on a separate EBA cohort of mice ($n=4$/time point). Treatment was on days 0–10 (alternative days), daily 8–10, or daily 12–14 ($n=4$/time point/treatment). Digital images of blistered skin were taken daily and analyzed for macroscopic assessment of blistering using Image Pro Plus, as previously described (Kopecki *et al.*, 2011a). Measurements of TEWL were obtained with a calibrated Vapometer evaporimeter (Delfin Technologies, Kuopio, Finland) on days 0–10 or 7–16 following the manufacturer's instructions. At day 12 or 16, mice were killed using CO_2 asphyxiation and cervical dislocation. Samples of back skin were collected in 10% buffered formalin for histological blister scoring as previously described (Kopecki *et al.*, 2011a). Additional samples of skin were collected for biochemical analysis and snap-frozen in liquid nitrogen. Strips of skin were assessed using tensiometry to determine skin strength using the Mecmesin Advanced Force Gauge AFG-100N (Mecmesin, Slinfold, UK) with a motorized test stand (Mecmesin M1000E, Mecmesin) at a speed of $3\,cm\,minute^{-1}$ following the manufacturer's instructions. Organs, tissues, and serum of wild-type mice with no EB that were treated with a single application of FnAb cream or IgG control cream were collected for biochemical analysis and for the detection of Flii and FnAbs.

Materials and methods for histology, immunohistochemistry, western blotting, FnAb detection ELISA, and statistical analysis may be found in supporting information, Supplementary materials and methods.

SUPPLEMENTARY MATERIAL

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

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