Kindlin-1 contributes to EGF-induced re-epithelialization in skin wound healing

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Abstract. The commercial use of epidermal growth factor (EGF) is extensive and has been shown to be effective for skin wound healing in clinical practice. There is evidence to indicate that the topical administration of EGF significantly accelerates re-epithelialization by promoting keratinocyte mitogenesis and migration following acute injury; however, the mechanisms involved remain to be elucidated. Thus, in this study, we focused on Kindlin-1, a four-point-one, ezrin, radixin, moesin (FERM)-domain-containing adaptor protein, and report its contribution to EGF-induced re-epithelialization in skin wound healing. In tissue samples, the expression of Kindlin-1 was induced upon EGF treatment compared to that in the natural healing group. In immortalized human keratinocytes (HaCaT cells), we further proved that Kindlin-1 was necessary for mediating EGF-induced activation signals, including integrin β1 activation, focal adhesion kinase (FAK) phosphorylation and actin re-organization, which finally led to enhanced cell proliferation and migration. These results indicate that Kindlin-1 is essential in EGF-induced re-epithelialization in skin wound healing and provide additional rationale for the clinical application of EGF in the treatment of acute wounds.

Introduction

Skin wound healing begins immediately following an injury and consists of several overlapping stages: hemostasis, inflammation, proliferation and remodeling (1). These phases proceed with well-organized interaction among various types of cells, including platelets, keratinocytes, fibroblasts, endothelial cells and macrophages.

Keratinocytes, the major cellular component of the epidermis, are critical to wound re-epithelialization. Under normal conditions, several layers of keratinocytes form the integumentary system, providing a physical barrier between the environment and the organism, thereby protecting the body from external agents and pathogens (2). Once injured, keratinocytes undergo migration, proliferation and differentiation in order to maintain the integrity of the skin barrier.

Stimuli that prompt keratinocyte activation include growth factors, cytokines and chemokines (3-5). Epidermal growth factor (EGF) may be one of the most well-characterized growth factors in skin wound healing. In acute wounds, EGF is mainly secreted by platelets, macrophages and fibroblasts and is upregulated within a short period of time following injury (6,7). The release of EGF stimulates epithelial cell migration and proliferation, thus contributing to re-epithelialization (8).

A great deal of research efforts have focused on understanding the mechanisms of EGF responses in keratinocytes. Increased integrin expression can be detected in EGF-stimulated corneal keratinocytes, and has been proven to be responsible for the downstream events triggered by EGF (9). As regards re-epithelialization, integrins serve as cell attachment receptors and mediators of cellular signaling. Compared to the other subunits, the β1 family integrins are relatively well established: the increased expression of α2β1, α3β1, α9β1, α5β1 and αVβ1 can be detected in the epidermis after wounding (10,11); the loss of β1 integrins in keratinocytes results in defective migration (12); the conditional deletion of the β1 subunit in the mouse epidermis has been shown to prevent re-epithelialization and inhibit the closure of excisional wounds (13). All these clues point to the vital role of β1 integrins in wound healing.

To fully understand integrin bidirectional signaling, numerous researchers have focused on integrin binding proteins, which are critical in bridging integrin to various signaling pathways. For example, talin and intergrin-linked kinases (ILK) (14). Recent studies reported that Kindlins, a protein family structurally related to talin, also act as adaptor molecules by i) cooperating with talin to activate integrin (15),

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and ii) linking integrin with the cytoskeleton, scaffolding and signaling proteins by interacting with ILK, and recruiting proteins to focal adhesions (16,17). Given such findings, we attempted to explore the potential role of Kindlin-1 in EGF-induced wound healing.

Kindlins are evolutionary conserved, FERM-domain-containing adaptor proteins which play a vital role in integrin activation (18,19). All three members of the Kindlin family can directly bind to the membrane distal NPxY motifs within cytoplasmic integrin β tails, recruit cytoskeletal proteins and therefore activate intracellular cell signal transduction, as well as integrin binding to extracellular ligands (20). Among the Kindlins, Kindlin-1 is widely expressed in murine and human tissues, including the skin, heart, lungs, liver, kidneys, colon, prostate, ovaries and pancreas, and is predominantly distributed in epithelia (21,22). Mutations in the Kindlin-1 gene have been shown to cause Kindler syndrome, a rare human disease characterized by a variety of skin abnormalities, including skin blisters, photosensitivity, mucosal erosion and skin cancer (23), indicating that Kindlin-1 is critical in maintaining the normal function of the skin. However, the functions of Kindlin-1 in skin wound healing are not yet fully understood, although it has been previously demonstrated that a deficiency in Kindlin-1 results in reduced cell adhesion and increased apoptosis in keratinocytes (24).

In the present study, we first demonstrate that Kindlin-1 is involved in the natural skin wound healing process and in particular, it contributes to EGF-induced re-epithelialization in skin wound healing. The use of shRNA targeting Kindlin-1 efficiently suppressed EGF-induced HaCaT activation, including migration and proliferation by suppressing integrin β1 activation, focal adhesion kinase (FAK) phosphorylation and actin re-organization. Our data not only provide new mechanistic insight into the regulation of re-epithelialization in skin wound healing, but also lead to an improved understanding of the molecular basis underlying the application of EGF in the treatment of wounds.

Materials and methods

Ethics statement. Animal experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, which conforms to the provisions of the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Fudan University, Shanghai, China. All surgerical procedures were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering.

Wound model. Balb/c mice (Slac Laboratory Animal Co., Ltd., Shanghai, China) were deeply anesthetized before shaving the dorsal hair and cleaning the exposed skin with 70% ethanol. Subsequently, 5 mm full thickness punch wounds were created through the skin and the panniculus carnosus on the dorsal surface (one wound each on the left and right side) of each mouse. For further experiments, including immunohisto-chemical anlaysis, real-time PCR and western blot analysis, the healed tissues were removed en bloc with a 7 mm punch to include a margin of unwounded skin at the indicated time intervals; the unwounded back skin tissues removed at day 0

were used as controls [in the following results, the controls are presented as 'normal', EGF(-) day 0 or EGF(+) day 0].

Experimental groups and treatment. A total of 60 Balb/c mice (8-9 weeks old) were randomly divided into 10 groups as follows: i) EGF(-) (wound model, but no EGF treatment) 1 day group; ii) EGF(-) 3 day group; iii) EGF(-) 7 day group; iv) EGF(-) 10 day group; v) EGF(-) 14 day group; vi) EGF(+) (wound model with EGF treatment) 1 day group; vii) EGF(+) 3 day group; viii) EGF(+) 7 day group; ix) EGF(+) 10 day group; and x) EGF(+) 14 day group. In the EGF(+) group, the wounds were treated with EGF (recombinant human epidermal growth factor derivative for external use, liquid (I), 2,000 IU/ml, S20010038; Shenzhen Watsin Genetech Co., Ltd. Shenzhen, China) once a day (spray once on each wound). In each group, 6 animals were included. At the end of the experiment, mice were sacrificed by cervical dislocation under anesthesia.

Immunohistochemistry and immunofluorescence staining. For immunohistochemical staining, fresh skin tissues were embedded by optimal cutting temperature (OCT) compound. Frozen sections of skin (10-μm-thick) were stained with rabbit polyclonal Kindlin-1 antibody (22215-1-AP; Proteintech Group Inc., Chicago, IL, USA) at a dilution of 1:100 overnight (4°C) and then incubated with Dako REAL™ EnVision™/HRP, Rabbit/Mouse (ENV) reagent (K5007; DAKO, Glostrup, Denmark). For tissue immunofluorescence staining (25,26), the frozen tissues were stained with F4/80 (a marker of macrophages), Keratin 6 (K6, a marker of keratinocytes), FSP1 (a marker of fibroblasts) and Kindlin-1 antibodies. For cell immunofluorescence staining, the cells were first fixed with 4% paraformaldehyde for 10 min at room temperature, rinsed with PBS and incubated in blocking solution for 60 min at room temperature. For Kindlin-1, activated integrin β1 [(integrin β1 (ac)] and phospho-FAK (p-FAK) labeling, the cells were incubated with the primary antibody for 2 h at room temperature, rinsed with PBS and incubated with the relevant secondary antibody for 1 h at room temperature; For actin staining, the cells were incubated with Phalloidin-iFluor 594 Conjugate (cat. no. 23122; 1:1,000; AAT Bioquest, Sunnyvale, CA, USA) followed by DAPI staining (Sigma, St. Louis, MO, USA). The following antibodies were used: rabbit polyclonal Kindlin-1 antibody (cat. no. 22215-1-AP; 1:50; Proteintech Group Inc.), mouse monoclonal activated integrin β1 antibody (cat. no. MAB2079Z; 1:300, Millipore, Bedford, MA, USA), mouse monoclonal Kindlin-1 antibody (cat. no. SAB4200465; 1:250; Sigma), rabbit monoclonal phospho-FAK (Tyr397) antibody (cat. no. EP2160Y; 1:400; Abcam, Cambridge, UK), DyLight 405 affinipure goat anti-rabbit IgG (H+L) (cat. no. A23120; 1:800; Abbkine, Los Angeles, CA, USA), DyLight 405 affinipure goat anti-mouse IgG (H+L) (cat. no. A23110; 1:800; Abbkine), Alexa Fluor 594 affinipure goat anti-mouse IgG (H+L) (cat. no. 115-585-003; 1:800; Jackson ImmunoResearch, West Grove, PA, USA), Alexa Fluor 594 affinipure goat anti-rabbit IgG (H+L) (cat. no. 111-585-003; 1:800; Jackson ImmunoResearch). Images were captured using a Nikon Eclipse 660 microscope (Nikon, Tokyo, Japan) or a confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). A total of 6 samples from each group was selected. For each sample, at least 4 fields were captured.

Cell culture and RNA interference-induced knockdown of Kindlin-1. The immortalized keratinocyte cell line, HaCaT, (KCB 200442YJ, Kunming Cell Bank of Chinese Academy of Science, Kunming, China), were kept in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum and 1% penicillin/streptomycin. To inhibit the expression of Kindlin-1 in the HaCaT cells, shRNA targeting Kindlin-1 [EGFP-Kindlin-1 shRNA (hu)] lentiviral particles (Genechem, Shanghai, China), a pool of viral particles containing 3 target-specific constructs that encode 19 nt shRNA designed to knock down Kindlin-1 expression in the HaCaT cells, were transduced into the cells according to the manufacturer's instructions. Control lentiviral particles (Genechem) containing a scrambled shRNA sequence, were used as a negative control. The knockdown efficiency was confirmed by western blot analysis.

Real-time PCR. Real-time PCR was performed using the SYBR Primer Ex Taq™ II kit (Takara, Dalian, China). Data were obtained using the StepOne plus system (Applied Biosystems, Foster City, CA, USA). The relative change in Kindlin-1 mRNA expression was determined by the fold change. The sequences of the primers used were as follows: Mouse Kindlin-1 sense primer (5'→3'), GGAGAGCAGCA GACAGAGAT; antisense primer (5'→3'), TGCTGAGGGGTG AAGAGAAG. Mouse GAPDH sense primer (5'→3'), GAGCG AGACCCCACTAACAT; antisense primer (5'→3'): TCTCCAT GGTGGTGAAGACA.

Western blot analysis. Western blot analysis was carried out as previously described (25). Briefly, tissues and cell lysates were collected, and the protein concentrations were determined using a BCA™ Protein Assay kit (Pierce, Chemical Co., Rockford, IL, USA). Proteins were separated by 10% SDS-PAGE and electro-transferred onto PVDF membranes (Millipore). The membranes were first incubated with the indicated primary antibodies overnight (4°C), followed by HRP-conjugated secondary antibodies for 2 h (room temperature). Blots were visualized using an enhanced chemiluminescence detection kit (Tiangen Biotech Co., Ltd. Beijing, China). The following antibodies were used: mouse monoclonal Kindlin-1 antibody (cat. no. SAB4200465; 1:1,000, Sigma), rabbit monoclonal integrin β1 antibody (cat. no. NB110-57123; 1:1,000, Novus Biologicals, Littleton, CO, USA), mouse monoclonal activated integrin β1 antibody (cat. no. MAB2079Z; 1:1,000, Millipore), rabbit polyclonal FAK antibody (cat. no. 3285; 1:1,000, Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit monoclonal Phospho-FAK (Tyr397) antibody (cat. no. 8556; 1:1,000, Cell Signaling Technology, Inc.), rabbit monoclonal GAPDH antibody (cat. no. 3683, 1:1,000; Cell Signaling Technology, Inc.). All experiments were repeated at least 4 times.

Induction of FAK activation in HaCaTs. Fibronectin (FN, PHE0023; Gibco, Frederick, MD, USA), was used to induce cell adhesion. Briefly, dishes were coated with FN (10 μg/ml, 37°C) overnight. Detached serum-starved control HaCaT cells and Kindlin-1 shRNA-transfected HaCaT cells were stimulated with or without EGF (10 ng/ml) for 15 min and were then subjected to FN-coated dishes. Cells were harvested at 30 min, 1 h and 3 h, respectively, and FAK phosphorylation was detected by western blot analysis.

Migration assay. Migration assay was performed using Transwell cell culture systems (3422; Corning, NY, USA), as previously described (26). Briefly, the HaCaT cells were added into the upper chamber. In order to verify the role of FAK in HaCaT cell migration, normal cells were first treated with or without FAK inhibitor, PF573228 (S2013; Selleck, Houston, TX, USA) (1 μM, 30 min) and then treated with or without recombinant human EGF (236-EG; R&D Systems, Minneapolis, MN,USA) (10 ng/ml) for 24 h. In order to investigate the role of Kindlin-1 in HaCaT cell migration, normal, Kindlin-1 shRNA and control shRNA cells were treated with or without recombinant human EGF (10 ng/ml) for 24 h. The infiltrating cells were stained with crystal violet (Beyotime, Shanghai, China), and cell numbers were counted from 5 wells. All experiments were repeated 3 times.

Cell proliferation assay. Cell proliferation was determined using the Cell Counting Kit-8 (CCK-8; Dojindo, Kunamoto, Japan) following the instruction manual. In brief, the cells were treated with or without EGF (10 ng/ml) for 24 h and then incubated with CCK-8 solution for 1.5 h. The absorbance was determined at 450 nm using a Universal Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT, USA). All assays were performed in triplicate, and the experiment was repeated 5 times.

Statistical analysis. The results are presented as the means ± SEM. Statistical analysis was performed using One-way ANOVA. Statistical significance was determined at the level of $P<0.05$.

Results

Dynamic expression of Kindlin-1 in EGF-induced skin wound healing in mice. In our initial set of experiments, we examined Kindlin-1 expression in skin tissues. Animals that underwent incisions were randomly divided into 2 main groups (with or without EGF treatment) and raised for different periods of time (Fig. 1A1). As shown in Fig. 1A2, the topical application of EGF improved the healing rate (72.73% vs. 90.54% on the 14th day). Kindlin-1 mRNA expression was assessed by real-time PCR. As shown in Fig. 1B, the mRNA expression of Kindlin-1 reached the highest level at 1 day post-injury. The administration of EGF resulted in the more rapid and prolonged increase of Kindlin-1 mRNA expression. Similar alterations were observed by western blot analysis (Fig. 1C1 and C2). The dynamic expression of Kindlin-1 suggested that it is involved in the process of skin wound healing and is very likely to contribute to EGF signaling.

Distribution of Kindlin-1 in skin wounds. The distribution of Kindlin-1 was detected by immunostaining. As shown in Fig. 2A, in normal skin, Kindlin-1 was mainly located in the epidermis with low intensity. During the natural healing process [EGF(-) group, Fig. 2B-D], strong Kindlin-1 staining was found 3 days after wounding, mainly distributed in the epidermis and sporadically in the new dermal fibroblasts (Fig. 2B). On the 7th day, moderate Kindlin-1-positive staining was observed in the regenerated epidermis, which decreased with the distance from the wound center (Fig. 2C). On the 14th day, only weak Kindlin-1 staining was observed in
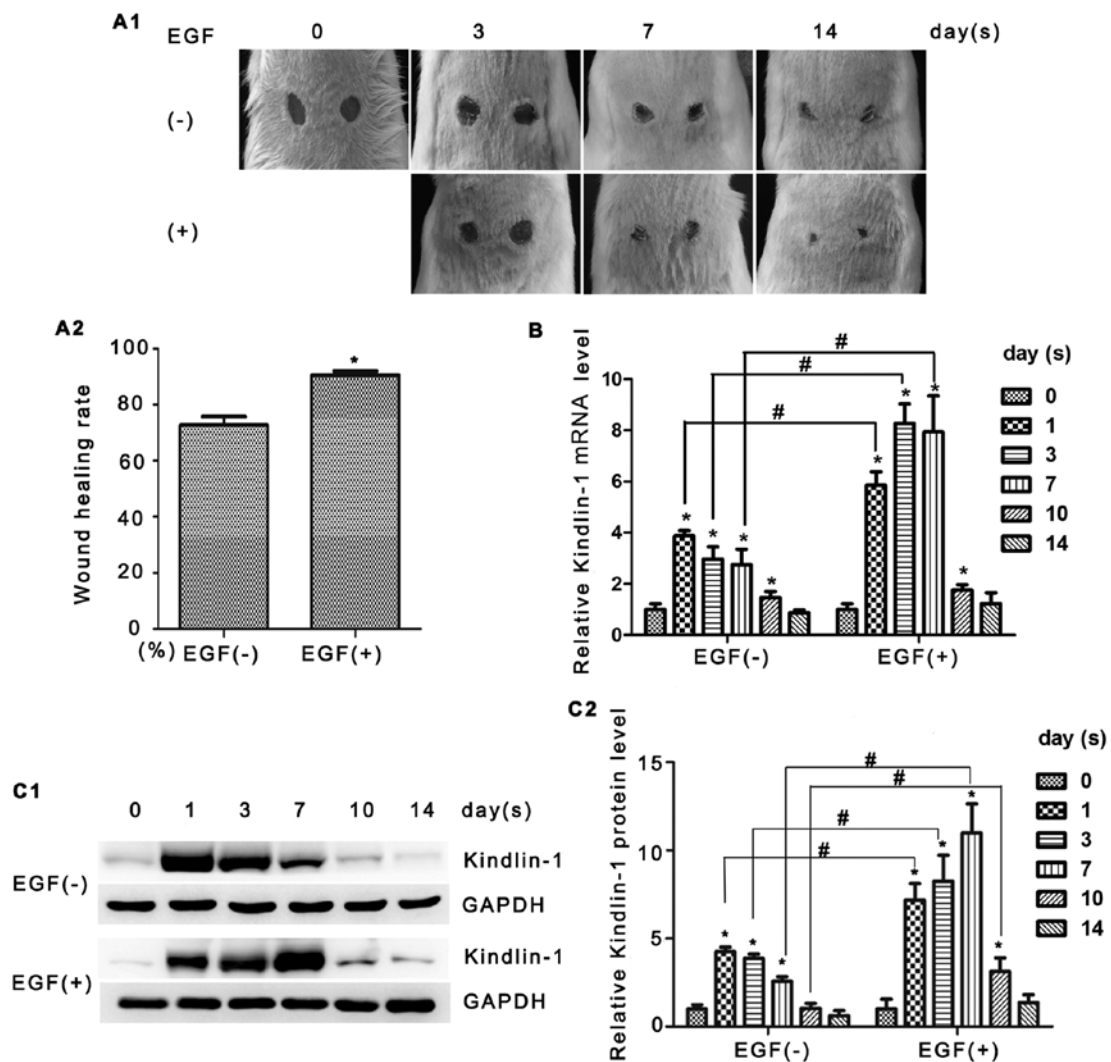
Figure 1. Dynamic expression of Kindlin-1 in skin wound healing in mice. Assessment of Kindlin-1 in skin wound healing in mice with or without EGF treatment. (A1)Construction of wound healing model in mice. (A2) Healing rate of EGF(-) and EGF(+) group at the 14th day. *P<0.05. (B1) Kindlin-1 expression was detected by real-time PCR. (C1) Kindlin-1 expression was detected by western blot analysis and with GAPDH as an internal control. (C2) Kindlin-1 expression was quantified and presented by the fold changes compared with the relative control [value of EGF(-) 0 days or EGF(+) 0 days]. *P<0.05, significant difference compared to the normal group [EGF(-) 0 days, EGF(+) 0 days]. #P<0.05.
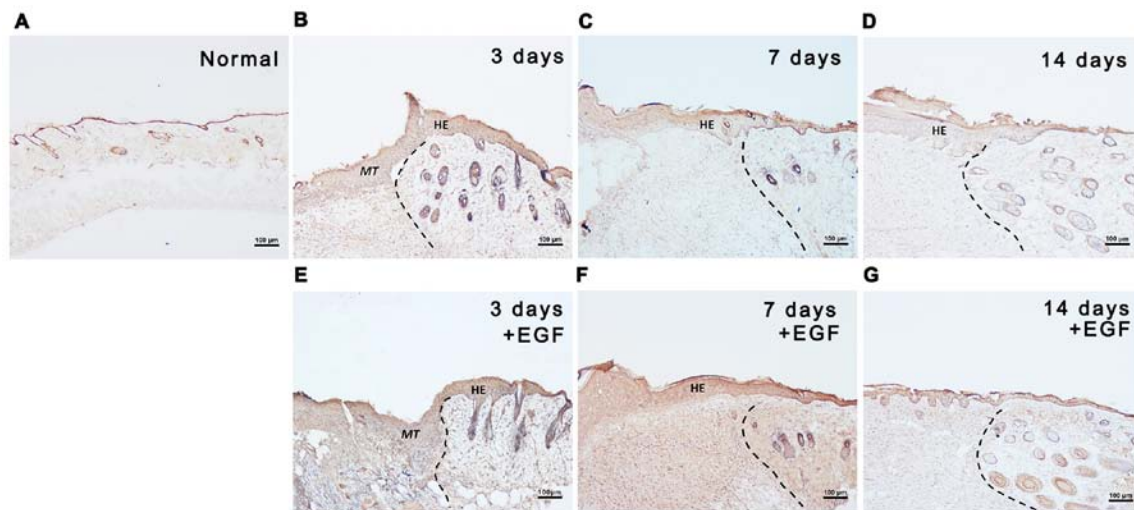

Figure 2. Distribution of Kindlin-1 in mouse skin tissues. Expression pattern of Kindlin-1 in mouse tissue slices was observed at the indicated time intervals after operation. (A) Normal; (B-D) EGF(-) 3 days, 7 days and 14 days, respectively; (E-G) EGF(+), 3 days, 7 days and 14 days, respectively. MT, migratory tongue; HE, hyperproliferative epithelium. The dotted line indicates the wound edge. Scale bar, 100 *μ*m.
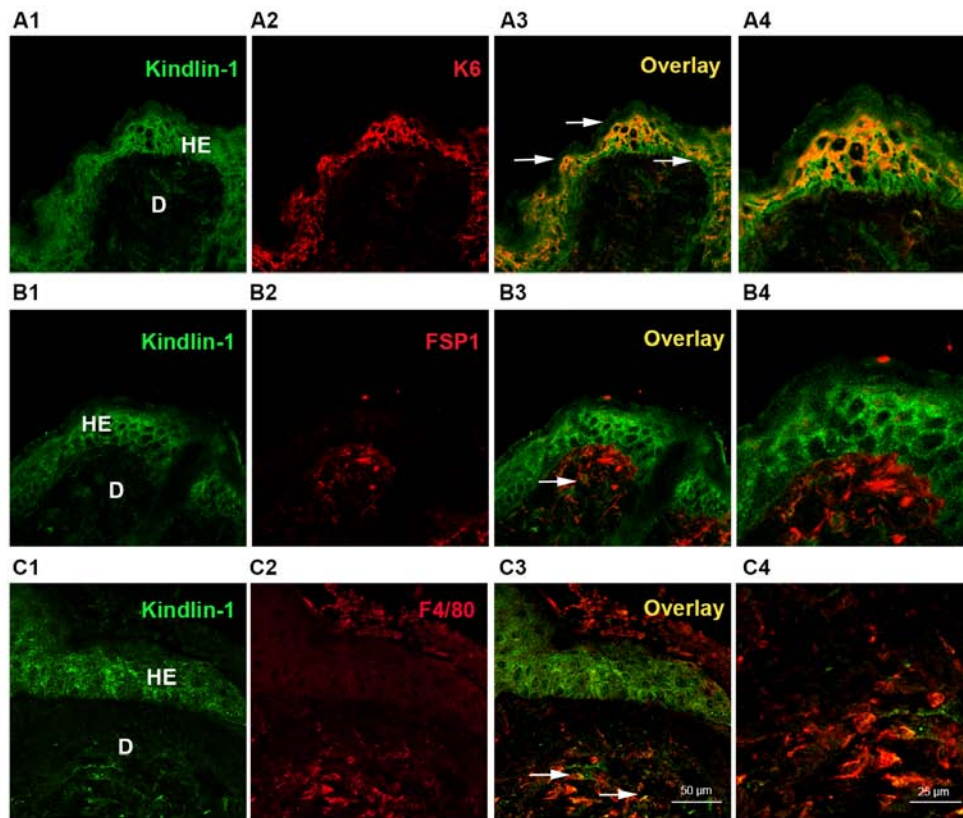
Figure 3. Cell location of Kindlin-1. Cell location of Kindlin-1 was observed in the EGF(+) 3 day group. Kindlin-1 (green) was double stained with K6 (A2-A3, red) FSP1 (B2-B3, red) and F4/80 (C2-C3, red); arrows indicate co-localization; scale bar, 50 μm. A4, B4 and C4 show an enlarged image; scale bar, 25 μm.

the wound epidermis (Fig. 2D). A more intense kindlin-1-positive signal was observed in the EGF(+) group (Fig. 2E-G); the most intense staining was found in the epidermal cells post-injury. Double labeling immunofluorescence (Fig. 3) further indicated that most of Kindlin-1 was located in the keratinocytes (Fig. 3A4), and to a lesser extent in fibroblasts (Fig. 3B4) and macrophages (Fig. 3C4). The specific accumulation of Kindlin-1 in keratinocytes encouraged us to further investigate the possible role of Kindlin-1 in re-epithelialization.

Expression of Kindlin-1 in EGF induces HaCaT cell activation in vivo. As mentioned above, keratinocytes are major cell components in re-epithelialization, EGF accelerates keratinocyte migration, induces rapid proliferation and exerts its effects in a paracrine manner (7,8). Therefore, in the following *in vitro* experiments, we used EGF and HaCaT cells to mimic the *in vivo* conditions of re-epithelization.

As shown in Fig. 4A1, in response to EGF stimuli, upregulated Kindlin-1 expression was detected 3 h after and sustained until the end of the observation (24 h, 2.47-fold vs. 0 h, Fig. 4A2). These data confirmed our hypothesis that Kindlin-1 may contribute to EGF-induced re-epithelialization through keratinocytes.

Kindlin-1 shRNA suppresses EGF-induced integrin β1-FAK activation and actin recruitment. It has been documented that there is a highly complex regulation of cell signaling in re-epithelialization (2). To further explore the signal transdution of EGF-Kindlin-1 in HaCaT cells, we employed Kindlin-1 shRNA

to search for downstream regulators. The interference and knockdown efficiency was confirmed by fluorescence microscopy (Fig. 4B) and western blot analysis (Fig. 4C). The Kindlin-1 knockdown population (Kindlin-1 shRNA) and the scrambled-RNAi population (Control shRNA) of HaCaT cells were used in the following assays.

Integrin β1 is one the best candidates of the downstream regulators either for its potential binding sites of Kindlin-1 or for its critical role in promoting re-epithelialization (27,28). In our study, EGF triggered integrin β1 expression and its activation was confirmed in Fig. 5A1-A4. Although Kindlin-1 knockdown yielded no obvious changes in integrin β1 protein expression (Fig. 5B1 and B3), reduced integrin β1 activation [integrin β1 (ac)] was observed in the EGF 0 h group, approximately 36% (Fig. 5B2, lane 3 vs. lane 2); a greater reduction was observed in the EGF 24 h group, an approximately 58% decrease when compared to the control shRNA group (Fig. 5B2, lane 6 vs. lane 5). Such findings were confirmed by immunofluorescence staining. Enhanced Kindlin-1 and integrin β1 (ac) staining was observed in the cells in the control shRNA group in response to EGF stimuli (Fig. 6B1 vs. A1 and B2 vs. A2), particularly at the cell peripheral sites. Intensive violet fluorescence in the inlays (Fig. 6B2 vs. A2) indicated the increased co-localization of Kindlin-1 and integrin β1 (ac) in the EGF-treated control shRNA-transfected cells. However in the cells transfected with Kindlin-1 shRNA, integrin β1 (ac) staining was less than that of the control shRNA-transfected cells, whether EGF was present in culture or not. These results implied that EGF evoked both the expression and binding
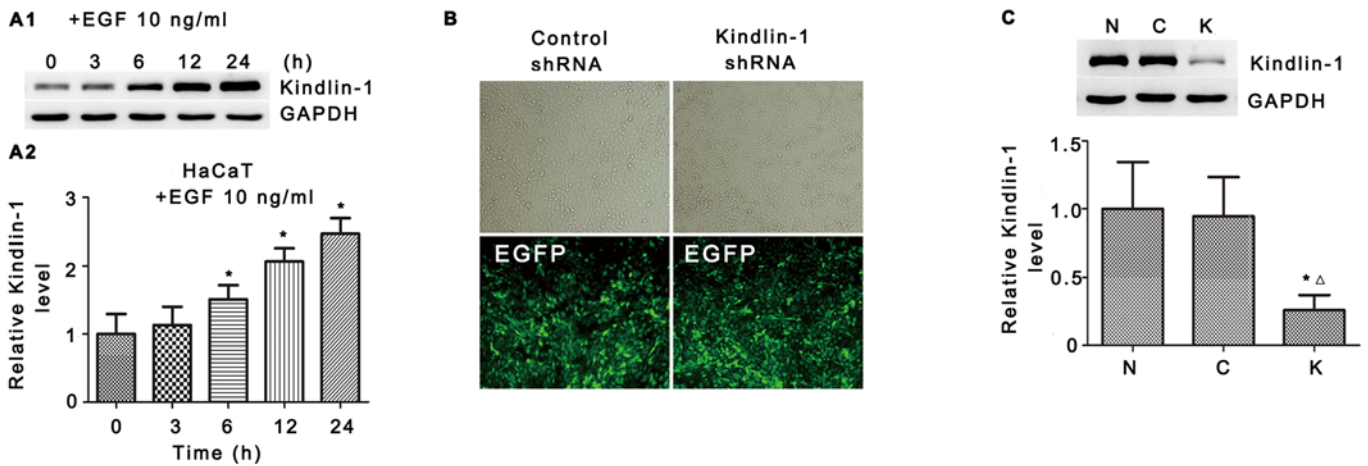
Figure 4. Expression of Kindlin-1 in EGF-stimulated HaCaT cells. (A1) Expression of Kindlin-1 in HaCaT cells in response to EGF (10 ng/ml) was assessed by western blot analysis. (A2) Blots were quantified and compared with GAPDH, the graphs show quantification data. Values were reported as the means ± SEM (n=4). *P<0.05, significant difference compared to the value at 0 h. (B-C) Knockdown efficiency of Kindlin-1 protein in HaCaT cells was determined by fluorescence microscopy and western blot analysis. Quantification data are shown in the graph (C). *P<0.05, significant difference compared with the normal group; △P<0.05, significant difference compared with the control shRNA group. EGF, epidermal growth factor; N, normal HaCaT cells; C, control shRNA-transfected HaCaT cells; K, Kindlin-1 shRNA-transfected HaCaT cells.
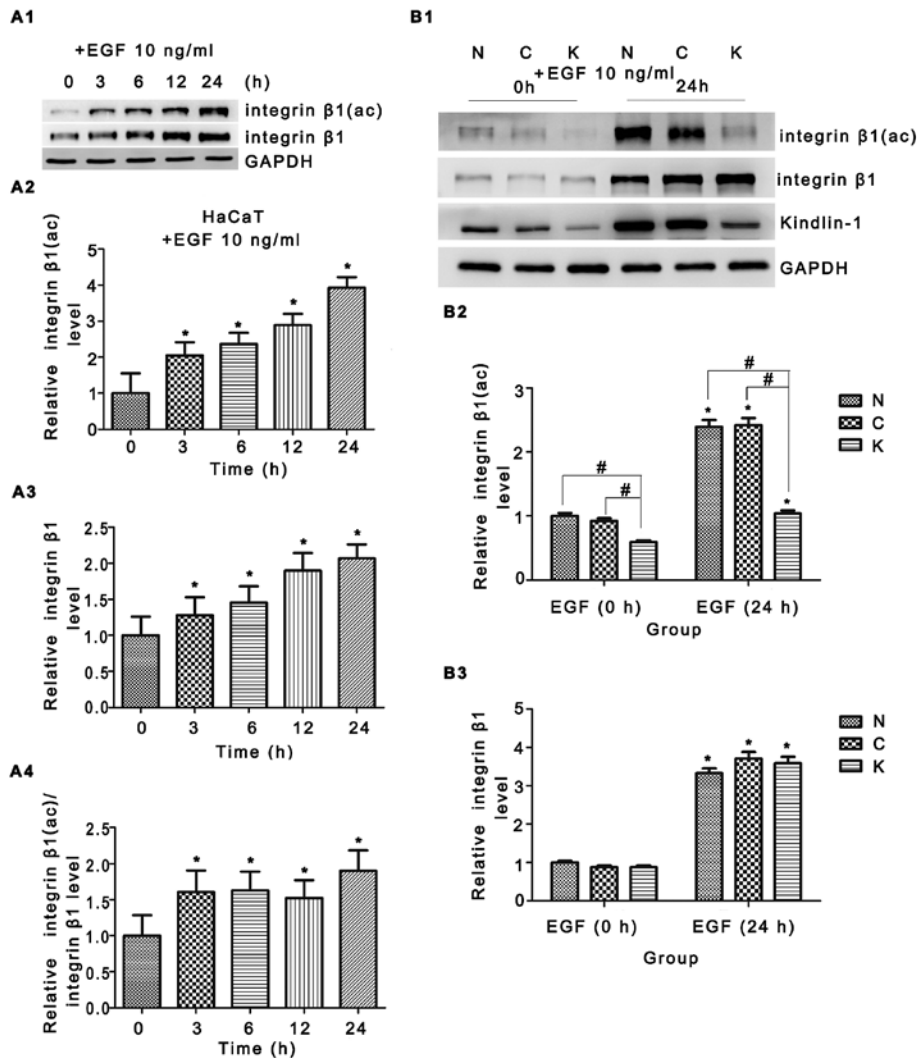

Figure 5. Kindlin-1 knockdown attenuates EGF-induced integrin β1 activation. (A1) Normal HaCaT cells were incubated with EGF (10 ng/ml) for the indicated periods of time, integrin β1 and its activated form were detected by western blot analysis and quantified in (A2 and A3), integrin β1 (ac)/ integrin β1 was quantified as shown in (A4). *P<0.05, significant difference compared to the value of the EGF (0 h) group. (B1) Normal, control shRNA- and Kindlin-1 shRNA-transfected HaCaT cells were incubated with EGF for 24 h, integrin β1 and its activated form were detected by western blot analysis and quantified as shown in (B3 and B2). *P<0.05, significant difference compared to the same type of cells in the EGF (0 h) group. #P<0.05. EGF, epidermal growth factor.
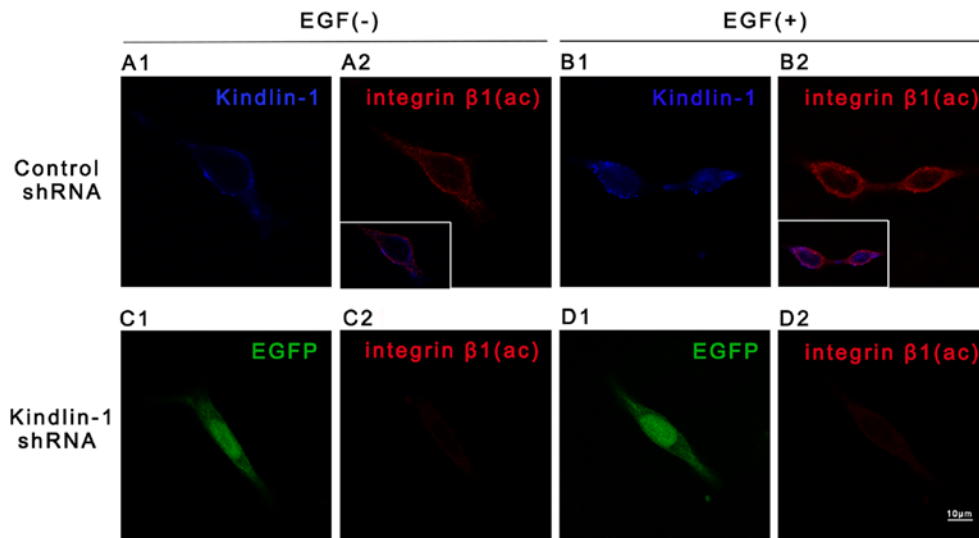
Figure 6. Activated integrin β1 staining in HaCaT cells. Cellular localization of activated integrin β1 (integrin β1 ac) and Kindlin-1 were observed using confocal microscopy. (A1 and A2) Kindlin-1 staining (blue) and integrin β1 (ac) (red) in control shRNA-transfected HaCaT cells without EGF stimuli. (B1 and B2) Kindlin-1 (blue) and integrin β1 (ac) (red) staining in control shRNA-transfected HaCaT cells exposed for 6 h to 10 ng/ml EGF. (C1 and C2) EGFP (green) and integrin) β1) (ac) staining (red) in Kindlin-1 shRNA-transfected HaCaT cells without EGF stimuli. (D1 and D2) EGFP (green) and integrin) β1) (ac) staining) (red) in Kindlin-1 shRNA-transfected HaCaT cells with EGF stimuli. Co-localization of integrin β1 (ac) and Kindlin-1 is shown in the inlays of panels A2 and B2 (violet). Scale bar, 10 μm. EGF, epidermal growth factor.
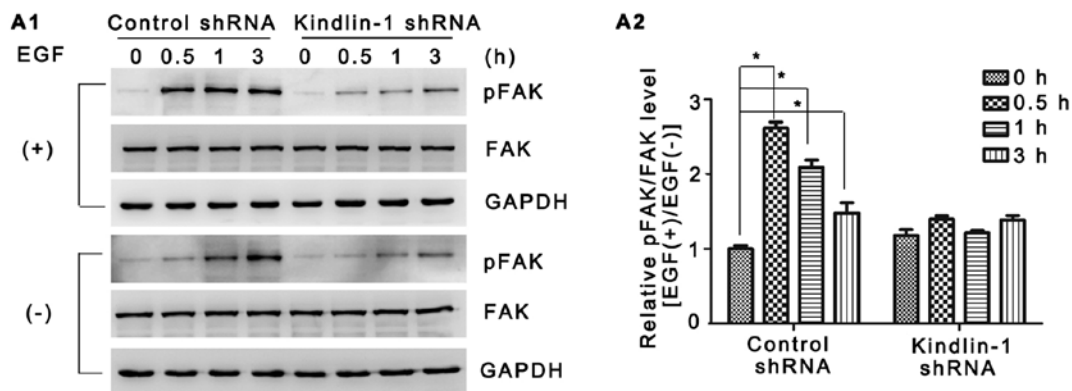

Figure 7. Kindlin-1 knockdown attenuates EGF-stimulated FAK phosphorylation in HaCaT cells. Detached serum-starved control HaCaT cells and Kindlin-1 shRNA-transfected HaCaT cells were stimulated with or without EGF (10 ng/ml) for 15 min and then subjected to FN-coated dishes for 30 min, 1 h and 3 h, respectively. (A1) phospho-FAK (p-FAK) and FAK levels were detected by western blot analysis and with GAPDH as an internal control. (A2) EGF induced p-FAK/FAK expression was presented by the fold changes compared with the value of control shRNA [EGF(+)/EGF(-), 0 h]. [*]P<0.05. EGF, epidermal growth factor; FAK, focal adhesion kinase.

of Kindlin-1 and integrin β1 (ac), while Kindlin-1 shRNA suppressed this effect. Together with the regulatory role of Kindlin-1 in integrin β1 trafficking (29), our data indicated that Kindlin-1 was very likely to be a mediator of EGF-induced integrin β1 signaling.

FAK is a widely expressed non-receptor tyrosine kinase which is responsible for multiple cell functions by mediating integrin signaling transduction (30-32). In our study, of note, although no obvious difference was observed in FAK expression between the groups, EGF-induced FAK activation was confirmed in the control shRNA-transfected HaCaT cells by both western blot analysis and immunofluorescence staining (Figs 7A1 and 8B2 vs. A2). In contrast, both adhesion-dependent FAK autophosphorylation (Figs 7A1 and 8C2 vs. A2,) and EGF-stimulated FAK phosphorylation were partly abolished in the Kindlin-1 knockdown group (Figs 7A1 and 8D2 vs. B2).

Fold changes in FAK phosphorylation following exposure to EGF were quantified as shown in Fig. 7A2. Furthermore, exposure to EGF increased the co-localization of Kindlin-1 and p-FAK in the control HaCaT cells (inlay of Fig. 8B2 vs. A2). Combined with our findings shown in Fig. 6, the above-mentioned data proved that the Kindlin-1-integrin β1-FAK complex formed at cell peripheral sites and suggested the existence of an EGF-Kindlin-1-integrin β1-FAK signaling pathway in HaCaT cells.

Actin is another candidate of downstream regulators of EGF-Kindlin-1 either for its indirect interaction to Kindlin-1 through migfilin, or its direct binding to the intracellular part of integrin β1 (18,33). Through the adjustment of its organization, the actin cytoskeleton contributes to integrin-mediated outside-in and inside-out signal transduction. Our results demonstrated that, following culture with EGF, more
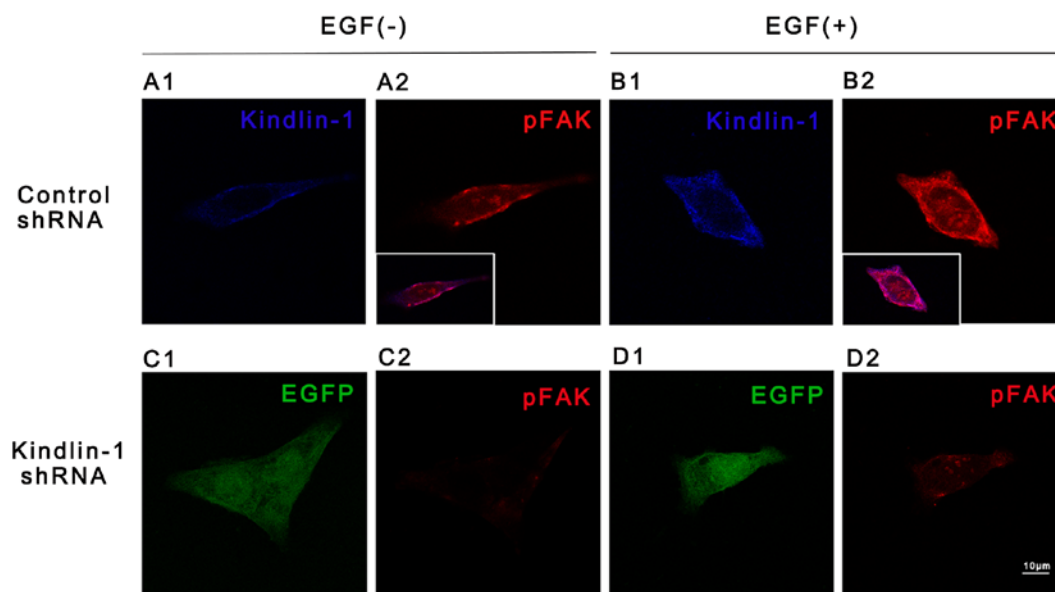
Figure 8. Phospho-FAK staining in HaCaT cells. Cellular localization of p-FAK and Kindlin-1 was observed using confocal microscopy. (A1 and A2) Kindlin-1 staining (blue) and p-FAK (red) in control shRNA-transfected HaCaT cells without EGF stimuli. (B1-B2) Kindlin-1 staining (blue) and p-FAK (red) in control shRNA-transfected HaCaT cells with 1 h exposure to 10 ng/ml EGF. (C1 and C2) EGFP (green) and p-FAK staining (red) in Kindlin-1 shRNA-transfected HaCaT cells without EGF stimuli. (D1 and D2) EGFP (green) and p-FAK staining (red) in Kindlin-1 shRNA-transfected HaCaT cells with EGF stimuli. Co-localization of p-FAK and Kindlin-1 are shown in the inlays of (A2 and B2) (violet). Scale bar, 10 μm. FAK, focal adhesion kinase; EGF, epidermal growth factor.
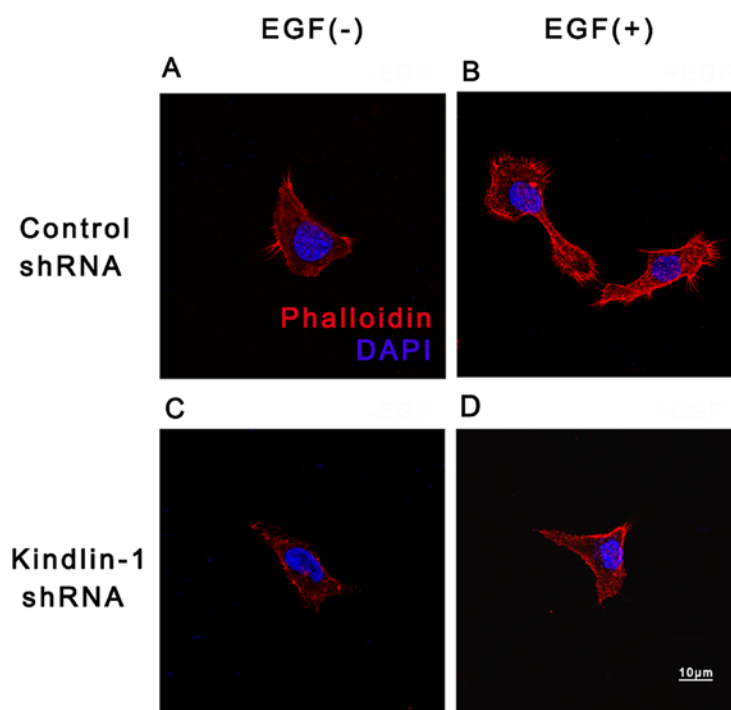

Figure 9. EGF-induced F-actin re-arrangement is disrupted by kindlin-1 shRNA. Staining of F-actin in HaCaT cells was by visualized with phalloidin staining (red) and confocal microscopy, DAPI counterstaining for cell nuclei (blue). (A) Control shRNA-transected HaCaT cells without EGF stimuli. (B) Control shRNA-transected HaCaT cells cultured with EGF for 6 h. (C) Kindlin-1 shRNA-transfected HaCaT cells without EGF stimuli. (D) Kindlin-1 shRNA-transfected HaCaT cells cultured with EGF for 6 h. Scale bar, 10 μm.

obvious cell actin was concentrated at cell extensions and/or cell borders (Fig. 9B) when compared with EGF(-) deprived control cultures (Fig. 9A). However, in the Kindlin-1 knockdown group, the actin network was disorganized and disrupted (Fig. 9D vs. C), suggesting that EGF-induced actin reorganization may be partly dependent on Kindlin-1.

Although previous studies have proven that there is no catalytic activity, Kindlin-1 may regulate cell functions by integrating and transmitting cell signals (16,17,34). In this study, in the presence of EGF, the potential role of Kindlin-1 in actin re-organization and its increased binding to integrin β1, as well as FAK, strongly suggested that Kindlin-1 may influence cell
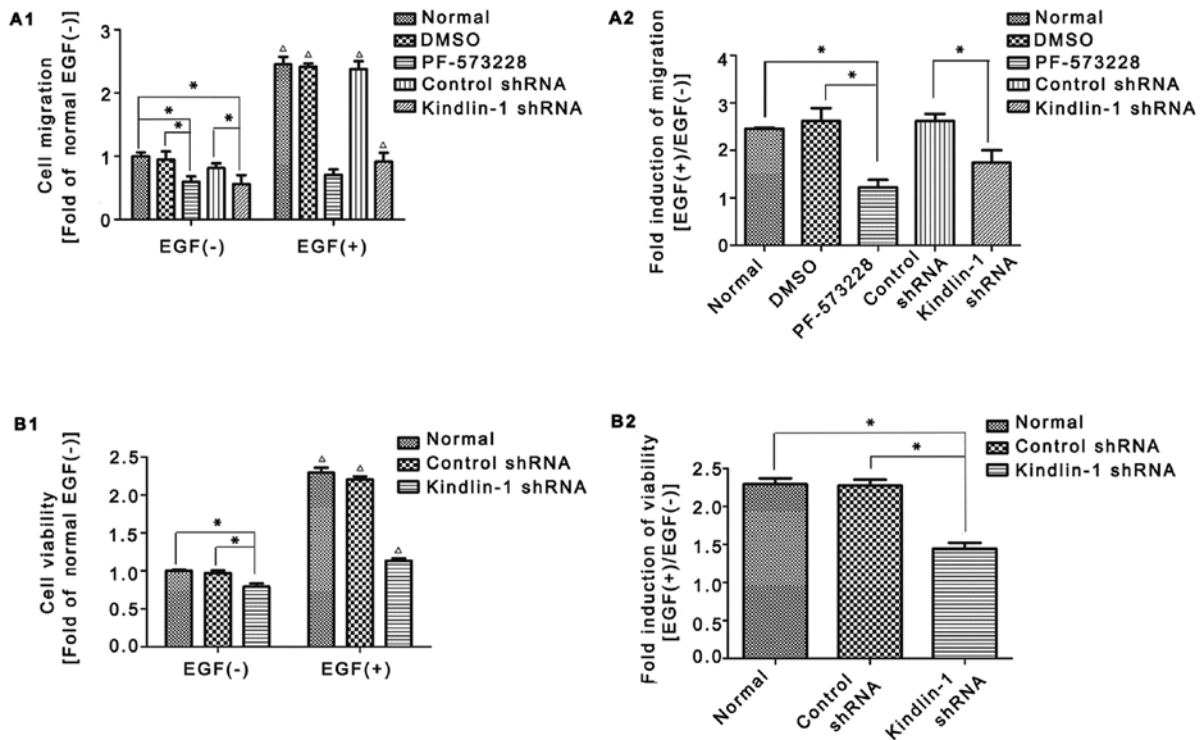
Figure 10. Kindlin-1 shRNA inhibits EGF-induced migration and proliferation of HaCaT cells. Normal, control shRNA- and Kindlin-1 shRNA-transected HaCaT cells were examined by Transwell assay, Normal cells were pre-treated with or without PF573228 (1 μM, 30 min) followed by EGF (10 ng/ml, 24 h). (A1) Cell migration was quantified by comparing with Normal [EGF(-)]. (A2) EGF-induced fold changes in cell migration in each group. After being incubated with or without EGF (10 ng/ml) for 24 h, Normal, control shRNA- and Kindlin-1 shRNA-transfected HaCaT cells were examine by CCK8 assay. (B1) Cell proliferation was quantified by comparing with normal group [EGF(-)]. (B2) EGF-induced fold changes in cell proliferation in each type of cells. *P<0.05 and $^\triangle$P<0.05, significant difference compared with the relative cells in the EGF(-) group. EGF, epidermal growth factor.

behavior in wound healing by directly or indirectly binding to signaling proteins and mediating protein interactions.

Kindlin-1 shRNA suppresses EGF-induced HaCaT cell migration and proliferation. To verify the above hypothesis, we then focused on examining the influence of Kindlin-1 on integrin β1 and FAK-related cell functions. During re-epithelization, keratinocytes migrate to the wound center and proliferate to fill the skin defect (35,36). Indeed, Kindlin-1 shRNA-mediated abnormal integrin regulation caused cell behavior defects: Transwell assay revealed that in the absence of EGF, PF-573228 suppressed EGF-induced HaCaT cell migration (Fig.10A1, lane 3 vs. lane 2); likewise, Kindlin-1 shRNA attenuated HaCaT cell migration compared to the control shRNA group (Fig. 10A1, lane 5 vs. lane 4). More marked differences were observed between the Kindlin-1 shRNA- and control shRNA-transfected cells following stimulation with EGF (Fig. 10A1, lane 10 vs. lane 9). The fold induction of cell migration following exposure to EGF was quantified as shown in Fig. 10A2 [EGF(+)/EGF(-)], Kindlin-1 shRNA restrained cell migration compared to the control shRNA group (approximately 35% reduction, lane 5 vs. lane 4), indicating Kindlin-1 shRNA partly abolished EGF induced migration.

Similar changes were observed for cell proliferation. Under normal conditions, slightly impaired cell proliferation was observed in the Kindlin-1 shRNA-transfected cells (Fig. 10B1, lane 3 vs. lane 2), while in the presence of EGF, a more rapid difference was observed between the groups (Fig. 10B1, lane 6 vs. lane 5). The fold induction of cell viability following

exposure to EGF was quantified as shown in Fig. 10B2 [EGF(+)/ EGF(-)]; Kindlin-1 shRNA repressed cell proliferation compared to the control shRNA group (approximately 45% reduction, lane 3 vs. lane 2).

Taken together, the inhibition of Kindlin-1 expression in HaCaT cells partially weakened EGF-induced integrin β1-FAK activation and the recruitment of cytoskeletal proteins, which finally led to impaired cell functions, including migration and proliferation. These results confirm the role of Kindlin-1 in transmitting EGF activation signals in skin wound healing.

Discussion

Multiple stimuli are responsible for inducing cell migration and proliferation during re-epithelialization, including an absence signal transmitted from the free edge of the wound and the increasing expression of growth factors (e.g., EGF and TGF). Clinical applications of growth factors in wound healing have been rapidly developed. In China, human recombinant EGF and basic fibroblast growth factor (bFGF) have been commercially available, and the topical administration of these growth factors has been proven to be effective for wound healing in clinical practice. Fully understanding the downstream signaling of EGF will allow for the more precise control of skin wound healing.

It has been reported that the major function of EGF in acute wounds is re-epithelialization (4). Keratinocytes are the main cell components of the epithelium, and also the main executors of re-epithelialization, considering the specific distribution of

Kindlin-1 in EGF-induced re-epithelialization. In the present study, we proposed a novel hypothesis (to the best of our knowledge) that in skin wound healing, EGF induced kindlin-1, which in turn activated intergrin β1 signaling to promote HaCaT keratinocyte activation.

By using full-thickness excisional wound models, the upregulated expression of kindlin-1 was observed in the epidermis. Indeed, the increased expression was not only detected in the EGF-treated group, but also in the untreated controls. This may at least partly be due to endogenic EGF. In the normal healing process, following injury, EGF is synthesized by platelets, lymphocytes, macrophages and fibroblasts, which acts on keratinocytes in a paracrine manner and finally contributes to keratinocyte activation (7,8). By contrast, in our study, the topical application of EGF directly acted on wounds at a higher concentration, thus accelerating the above-mentioned bio-effects. This may explain why the more rapid and durable upregulation of Kindlin-1 was found in the EGF-treated group (Fig. 1).

Considering the expression of Kindlin-1 in the epidermis and its enrichment in response to EGF, we then sought to explore its role in re-epithelialization, particularly in keratinocyte bioactivity. It has been demonstrated that Kindlins are co-activator of integrins (37). Evidence of the role of integrins in cell signal transmitting has accumulated. In the wound epidermis, intergrin β1 has been reported to serve as a bidirectional signal transduction molecule by binding to various signaling effectors directly or indirectly (38). These intergrin β1-related protein complexes convey signals, regulating gene expression and cell behavior, such as cell proliferation, migration and adhesion, as well as cell survival (39). Based on such observations, we hypothesized that Kindlin-1 may affect cell bioactivity through integrin β1. As expected, EGF stimuli evoked integrin β1 activation in HaCaT keratinocytes, and the blockade of Kindlin-1 led to decreased integrin β1 activation compared to the normal and control group (Fig. 5).

The activation of integrins allows the binding of intracellular signaling molecules, such as FAK, Src, p130C and other structural proteins. Up to 180 different scaffold and signaling proteins have been reported to be included in this network (40). As regards FAK for example, integrin activation can promote the combination of integrin and the ECM ligand and helps to form focal adhesion, which recruits focal adhesion proteins, including FAK. The β subunit of integrins can bind to the N-terminal regions of FAK to induce a structural change. FAK then switches to an active state (autophosphorylation of Tyr397) and triggers the downstream signaling pathway (41). FAK activation often occurs as a result of growth factor stimulation (42,43). In this study, we also observed FAK phosphorylation in response to EGF stimuli in serum-starved normal HaCaT cells, (Fig. 7), which is consistent with the study by Kim and Kim (44). However, in the Kindlin-1 shRNA group, the upregulation of FAK was attenuated. One reason may explain this phenomenon: in nomal HaCaT cells, integrin-mediated cell adhesion through focal adhesion provided the signaling transduction platform to activate FAK, and EGF accelerated this process. However, in Kindlin-1 shRNA-transfected HaCaT cells, the depletion of Kindlin-1 resulted in the impaired activation of intergrin β1, thus affecting the subsequent 'platform' foundation and FAK activation.

The activation of integrin-FAK and changes in actin conformation are related to several keratinocyte functions associated with wound healing. In this study, we first evaluated the role of Kindlin-1 in EGF-induced HaCaT cell migration and proliferation, two striking features of keratinocytes during re-epithelialization. Our results revealed that Kindlin-1 inhibition significantly attenuated EGF-induced HaCaT cell migration (Fig. 10A1 and A2) and proliferation (Fig. 10B1 and B2). These results indicated the existence of an EGF-Kindlin-1-integrin β1-FAK axis in triggering HaCaT cell activation.

It worth determining whether Kindlin-1 alone fulfilled the above function or cooperated with other molecules. In a previous study, Kindlin-2, another member of the Kindlins, was implicated in wound closure and tissue repair by acting on fibroblasts (45). Moreover, functional similarities between Kindlin-1 and Kindlin-2 have been reported, as they are localized in epidermal keratinocytes and have overlapping functions in adhesion and survival (46). Thus, Kindlin-1 and Kindlin-2 may function together in keratinocyte integrin activation during wound healing. However, this hypothesis and the exact mechanisms warrant further investigation.

In conclusion, our data provide evidence that in HaCaT keratinocytes, Kindlin-1 acts on integrin β1, FAK and actin to trigger cell activation. The inhibition of Kindlin-1 expression in HaCaT cells significantly attenuates the EGF-induced migration and proliferation. Given the essential role of keratinocyte activation in wound healing, Kindlin-1 may be a promising therapeutic target to promote wound healing.

Acknowledgments

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