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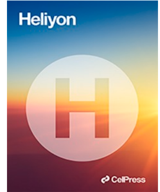

Research article

# Electric fields reverse the differentiation of keratinocyte monolayer by down-regulating E-cadherin through PI3K/AKT/Snail pathway

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ABSTRACT

Re-epithelialization is an important step in skin wound healing, referring to the migration, proliferation, and differentiation of keratinocytes around the wound. During this process, the edges of the wound begin to form new epithelial cells, which migrate from the periphery of the wound towards the center, gradually covering the entire wound area. These newly formed epithelial cells proliferate and differentiate, ultimately forming a protective layer over the exposed dermal surface. Wound endogenous electric fields (EFs) are known as the dominant factor to facilitate the epidermal migration to wound center. However, the precise mechanisms by which EFs promote epidermal migration remains elusive. Here, we found that in a model of cultured keratinocyte monolayer in vitro, EFs application reversed the differentiation of cells, as indicated by the reduction of the early differentiation markers K1 and K10. Genetic manipulation confirmed that EFs reversed keratinocyte differentiation through down-regulating the E-cadherin-mediated adhesion. By RNA-sequencing analysis, we screened out Snail as the transcription suppressor of E-cadherin. Snail knockdown abolished the down-regulation of E-cadherin and the reversal of differentiation induced by EFs. KEGG analysis identified PI3K/AKT signaling for Snail induction under EFs. Inhibition of PI3K by LY294002 diminished the EFs-induced AKT activation and Snail augmentation, largely restoring the level of E-cadherin reduced by EFs. Finally, in model of full-thickness skin wounds in pigs, we found that weakening of the wound endogenous EFs by the direction-reversed exogenous EFs resulted in an up-regulation of E-cadherin and earlier differentiation in newly formed epidermis in vivo. Our research suggests that electric fields (EFs) decrease E-cadherin expression by suppressing the PI3K/AKT/Snail pathway, thereby reversing the differentiation of keratinocytes. This discovery provides us with new insights into the role of electric fields in wound healing. EFs intervene in intracellular signaling pathways, inhibiting the expression of E-cadherin, which results in a lower differentiation state of keratinocytes. In this

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state, keratinocytes exhibit increased migratory capacity, facilitating the migration of epidermal cells and wound reepithelialization.

## 1. Introduction

Re-epithelialization stands as the foremost imperative phase in skin wound healing, entailing the migration, proliferation, and differentiation of keratinocytes at the wound periphery to construct a protective layer on the denuded dermal surface [1,2]. This process is orchestrated by an array of physical and chemical factors within the wound microenvironment, where endogenous electric fields (EFs) are purportedly instrumental in directing the collective migration of keratinocytes towards the wound center [3,4]. Endogenous electric fields (EFs) rapidly appear after an injury as a result of the collapse of *trans*-epithelial potentials. The wound center becomes more negatively charged compared to the surrounding tissue, acting as the cathode of the endogenous EF until re-epithelialization is complete [4]. Initial determination of endogenous wound electric fields traces back more than 150 years to the work of the German physiologist [5]. Subsequent advancements in techniques, such as the utilization of glass microelectrodes and vibrating probes, have consistently confirmed the presence of endogenous wound electric fields across various wound types. Notably, a substantial outward current of 4μA/cm$^2$-2 μA/cm$^2$ was observed at the wound edges of both rat cornea and human skin in previous studies [6]. In our previous work, we found that there was an endogenous wound electrical potential of about 70 mV in the normal non-electrically stimulated wound surface of the pig wound, and the electric field direction was from the wound margin to the wound center [7]. In vitro investigations have underscored the role of EFs in guiding the collective migration of keratinocytes through CD9-mediated ADAM17-driven EGFR signaling [8,9]. Nevertheless, the in vivo migration of keratinocytes as a coherent sheet entails a highly coordinated process, marked by intricate cellular changes favoring a motile phenotype, including the relaxation of cell-cell contacts, alterations in cell morphology, and down-regulation of differentiation markers such as keratin 1 and 10 [10,11]. Despite considerable attention being devoted to EFs-induced keratinocyte migration, the extent to which wound EFs contribute to the phenotypic changes of keratinocytes during re-epithelialization remains largely elusive.

High motility of cells is often associated with a low differentiation state [12]. We have discovered that CD9 regulates the transition between keratinocyte differentiation and motility by facilitating the recruitment of E-cadherin to the plasma membrane [13]. E-cadherin, the main member of the cadherin family in keratinocytes, facilitates cell-to-cell adhesion throughout all epidermal layers. It is known that the level of E-cadherin decreases in the newly formed epidermis, thereby loosening cell-cell adhesions and promoting epidermal migration within wounds [14,15]. Nonetheless, E-cadherin-mediated adhesion is not dispensable for the migration of keratinocyte sheets, as demonstrated by the abolition of collective migration with E-cadherin blocking antibodies [16]. Additionally, numerous studies have highlighted the importance of E-cadherin-mediated adhesion in cell differentiation [17,18]. Early differentiation of keratinocytes induced by contact inhibition can be hindered by E-cadherin antibody or E-cadherin dominant negative mutants [17]. Furthermore, deletion of E-cadherin leads to impaired keratinocyte terminal differentiation in mice [19]. Thus, E-cadherin-mediated adhesion plays a dual regulatory role in wound re-epithelialization, governing both the migration and differentiation of keratinocytes. In a prior investigation conducted by our team, we demonstrated that in a pig wound healing model, the application of exogenous EFs in the same direction as the wound's endogenous EFs reduced the protein level of E-cadherin in the newly formed epidermis, thereby promoting healing [20]. This finding suggests that wound EFs may directly regulate E-cadherin adhesion, thereby contributing to a low differentiation state in the newly formed epidermis, favoring epidermal migration in wound healing.

Here, utilizing models of cultured keratinocyte monolayers and pig wound healing in vivo, We investigated the role of EFs in regulating keratinocyte differentiation and elucidated their molecular mechanism. Our investigation revealed that the application of EFs decreased the protein level of E-cadherin and reversed the differentiation state in cultured keratinocyte monolayers, as evidenced by the reduction of early differentiation markers K1 and K10. Genetic manipulation confirmed a correlation between E-cadherin and keratinocyte differentiation, and the enhanced differentiation observed in E-cadherin-overexpressed cultures could also be attenuated by EFs, suggesting a significant role for EFs in regulating keratinocyte differentiation through the reduction of E-cadherin-mediated adhesion. Through RNA-sequencing, genetic knockdown, and subsequent inhibition experiments, we identified Sail as the transcription suppressor of E-cadherin that was upregulated through PI3K/AKT signaling under the influence of EFs. Finally, counteracting the wound's endogenous EFs by applying direction-reversed exogenous EFs resulted in the upregulation of E-cadherin and earlier differentiation in the newly formed epidermis in pig wounds, providing in vivo evidence for the regulatory role of EFs in keratinocyte differentiation.

## 2. Methods

### 2.1. Cell culture

Using HaCaT cells provided by the Cell Bank of the Chinese Academy of Sciences in Beijing, we cultured cells in RPMI-1640 medium (Cellgro; Corning, Inc.) supplemented with 10 % FBS(HyClone; Cytiva), Penicillin and streptomycin (Cellgro; Corning, Inc.). The culture environment was maintained in a humidified incubator at 37 °C with 5 % CO2. Subsequently, a monolayer of HaCaT cells was created in cell culture dishes using a PDMS template with rounded rectangular patterns. Prior to cell seeding, the template patterns were filled with DMEM to prevent bubble formation. Then, 200 μl of cell suspension with a density of 1 × 10ˆ6 cells/ml was pipetted onto the PDMS template. After approximately 1 h of cell settling and attachment to the substrate, any remaining cells were

gently removed by aspiration. The culture was then incubated for 12 h to allow the formation of a fused monolayer with mature cell connections. Prior to mounting onto a microscope, the PDMS template was carefully removed, and a second layer of PDMS was placed to seal the observation area.

### 2.2. EFs stimulation and imaging of cells

The cells were first placed in an electric field chamber to receive Electric Field (EF) stimulation as described previously [21]. Subsequently, the cells were immersed in Steinberg's solution, which consisted of 60 mM sodium chloride, 0.7 mM potassium chloride, 0.8 mM magnesium sulfate, 0.3 mM calcium nitrite tetrahydrate, and 1.4 mM tris(hydroxymethyl)aminomethane hydroxide, with a pH of 7.4. Next, EFs were applied in the electric field chamber using two electrodes and two agar bridges.

### 2.3. Small interfering RNA transfection, recombinant adenoviral vectors and drug treatments

Human E-cadherin small interfering RNA (siRNA-E-cad 2368), human Snail small interfering RNA (siRNA-Snail 535), and control siRNA (siRNA-NC) were provided by Shanghai GenePharma. Subsequently, siRNAs were transfected into cells using HiPerFect transfection reagent, with 100 pmol of each siRNA transfected into one well of a 6-well plate. Transfected cells were then incubated at 37 °C for 2 days.Ad-E-cadherin and the E-cadherin mimic vector Ad-GFP were provided by Shanghai GeneChem. HaCaT cells were seeded into 6-well plates and infected with Ad-E-cadherin-GFP. Subsequently, cells were infected with Ad-E-cadherin-GFP and the E-cadherin mimic vector Ad-GFP for 48 h.Transfection efficiency was observed via fluorescence microscopy and western blotting experiments.

To investigate potential mechanisms, cells were pretreated with LY294002 (PI3K inhibitor; Sigma, 20 mM) for 12 h before exposure to EFs.

### 2.4. Immunofluorescence

Cells were initially fixed with 4 % paraformaldehyde at room temperature for 15 min, then permeabilized for 15 min and washed to eliminate any residual agents.Subsequently, cells were blocked with serum to prevent nonspecific binding. Then, primary antibodies (K1, K10, and E-cadherin) were sequentially applied, followed by secondary antibodies and DAPI staining to label the proteins of interest.

### 2.5. Western blots analysis

First, the cell monolayer was lysed using lysis buffer after washing with PBS, subjected to sonication, and finally centrifuged at 4 °C. The supernatant was extracted, and the protein concentration was determined using the Pierce BCA Protein Assay Kit.25 μg of protein from each sample was loaded onto a SDS gel, separated by electrophoresis, and transferred onto a PVDF membrane. Following blocking on the PVDF membrane for 1 h, primary antibodies were applied at a diluted concentration and incubated overnight at 4 °C. This was succeeded by a 1-h incubation with the corresponding secondary antibodies at room temperature. After washing, signals were detected using a molecular imager and chemiluminescent reagents, and grayscale quantification of the images was performed using ImageJ software.

The primary antibodies used in this experiment included: K1 (Abcam), K10(Abcam), E-cadherin(Abcam), Snail(Abcam), PI3K, p-PI3K(CST), AKT, *p*-AKT(CST), and GAPDH(CST).

### 2.6. RNA sequence

HaCaT cells were randomly divided into EFs (Electric Fields) group and no EFs group. After washing with PBS, cells were collected to extract total RNA, with two biological replicates prepared for each group, consisting of three samples each. RNA extraction was performed using the RNAprep pure Cell/Bacteria Kit (Tiangen Biotech), and cDNA fragment amplification was carried out according to the manufacturer's instructions for the kit.The final RNA-seq libraries were amplified using phi29 and sequenced using the BGISEQ-500 sequencer with single-end 50-cycle reads.

### 2.7. Animals

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Army Medical University. This study involved Bama miniature pigs aged 9–12 weeks, weighing between 15 and 20 kg, with good skin condition. Prior to the experiment, the pigs were acclimated in experimental cages for 72 h. Anesthesia was induced using inhalation of 1–3 vol% isoflurane (246885U, Abbott, China). During the experiment, pulse oximetry ear sensors were periodically used to measure oxygen saturation and heart rate to ensure safety. After the procedures were completed, The pigs were moved to enclosures and monitored throughout the recovery from anesthesia.

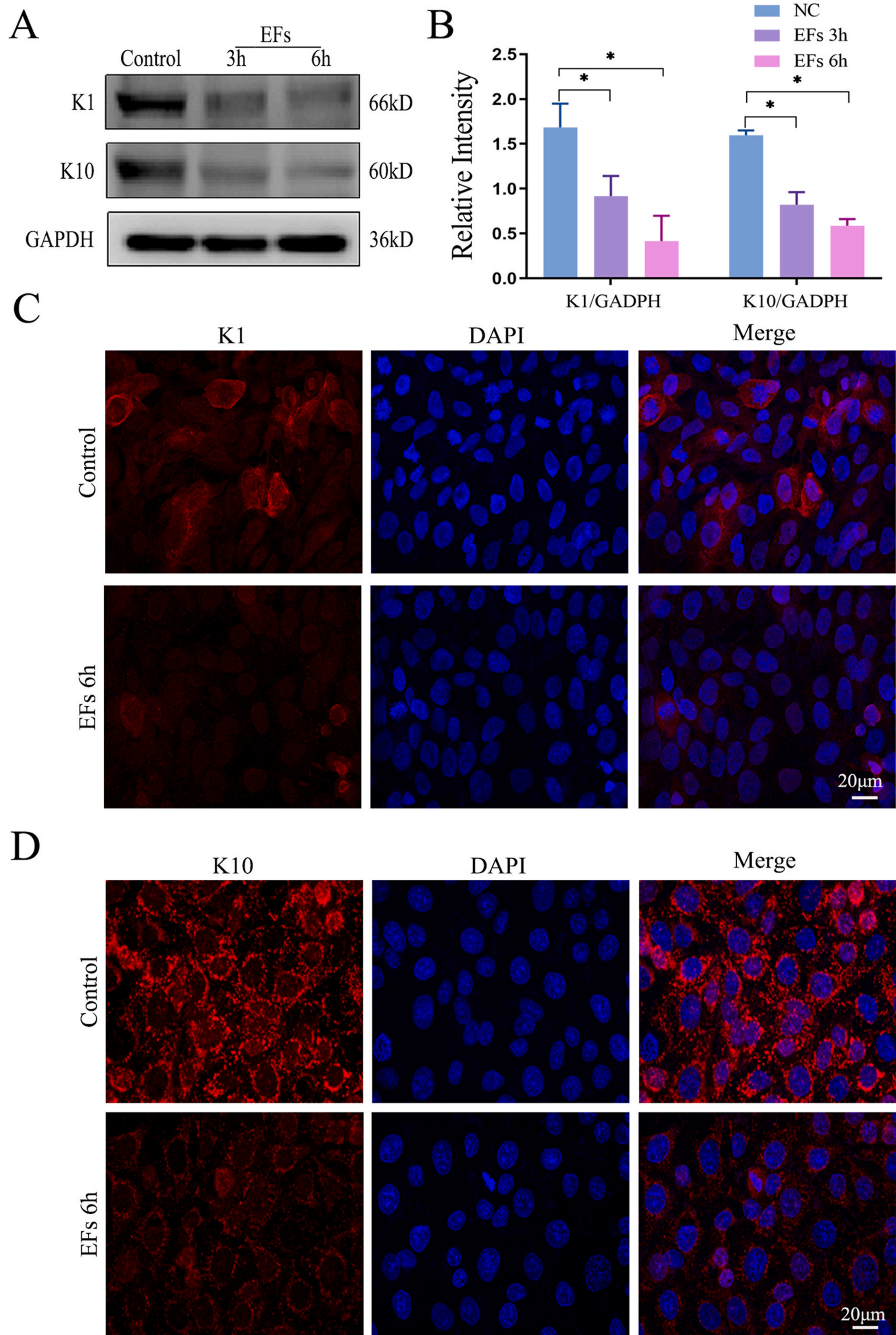


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**Fig. 1.** EFs reversed the differentiation of keratinocyte monolayer. (A, B) The protein levels of K1 and K10 in HaCaT cell monolyaer when treated with no EFs (control), EFs 3h and EFs 6h were tested by Western blot and the results were quantified by relative intensity. The data was shown as the mean ± SEM (n = 3). *, $p < 0.05$ compared with no EFs group. (C, D) The protein levels and distribution of K1 and K10 in cells under no EFs and electrified for 6h were observed by immunofluorescence. Bar = 20 μm.

### 2.8. Surgical approaches and direction-reversed exogenous EFs application

As described in our previous study [20], after positioning the pigs in the prone position, a centrally symmetric 30 × 30 cm skin area was selected as the surgical preparation zone. In this area, symmetrical circular full-thickness skin defects with a diameter of 3 cm were created on both sides. Ring electrodes were placed at the edges of the wounds, and cylindrical electrodes were placed in the center, with wires connecting the electrodes to a power source. To attenuate the endogenous electric field (90 mV/mm) within the wound, an exogenous electric field of 100 mV/mm was applied in the opposite direction of the endogenous field. For the control group, the wounds had electrodes placed but were not connected to the power source.

In total, 10 pigs were randomly assigned to the experiment. Fourteen days post-surgery, Photographs of the wounds were captured, and samples of wound tissue were collected for subsequent analysis.

### 2.9. Staining procedure

The tissue samples were first fixed in 4 % paraformaldehyde for one day, followed by routine paraffin embedding and sectioning. The sections were then subjected to Hematoxylin and Eosin (H&E) staining and immunohistochemistry (IHC) staining.For immunohistochemistry staining, the sections were blocked with serum at room temperature for 1 h and then incubated with the appropriate primary antibody overnight. After washing with buffer, the sections were incubated with a secondary antibody for 2 h, followed by the addition of 3,3′-diaminobenzidine (DAB) for color development. Once staining was complete, images of the tissue samples were captured under a microscope and analyzed using ImageJ software.

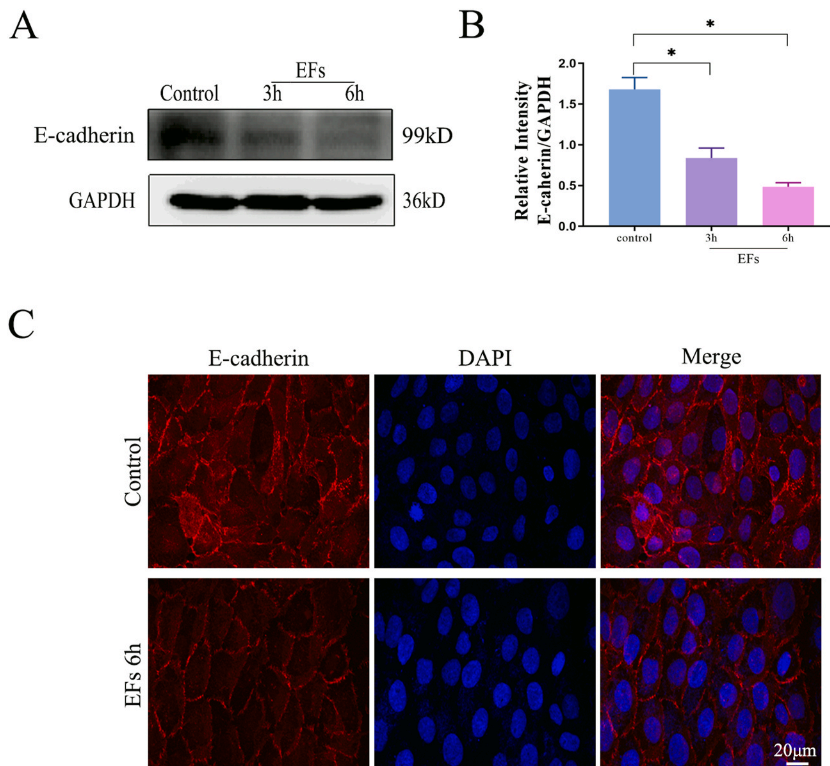


**Fig. 2.** EFs downregulated the E-cadherin protein level of keratinocyte monolayer. (A, B) The protein levels of E-cadherin in HaCaT cell monolyaer when treated with no EFs (control), EFs 3h and EFs 6h were tested by Western blot and the results were quantified by relative intensity. The data was shown as the mean ± SEM (n = 3). *, $p < 0.05$ compared with no EFs group. (C) The protein level and distribution of E-cadherin in cells under no EFs and electrified for 6h were observed by immunofluorescence and bar = 20 μm.

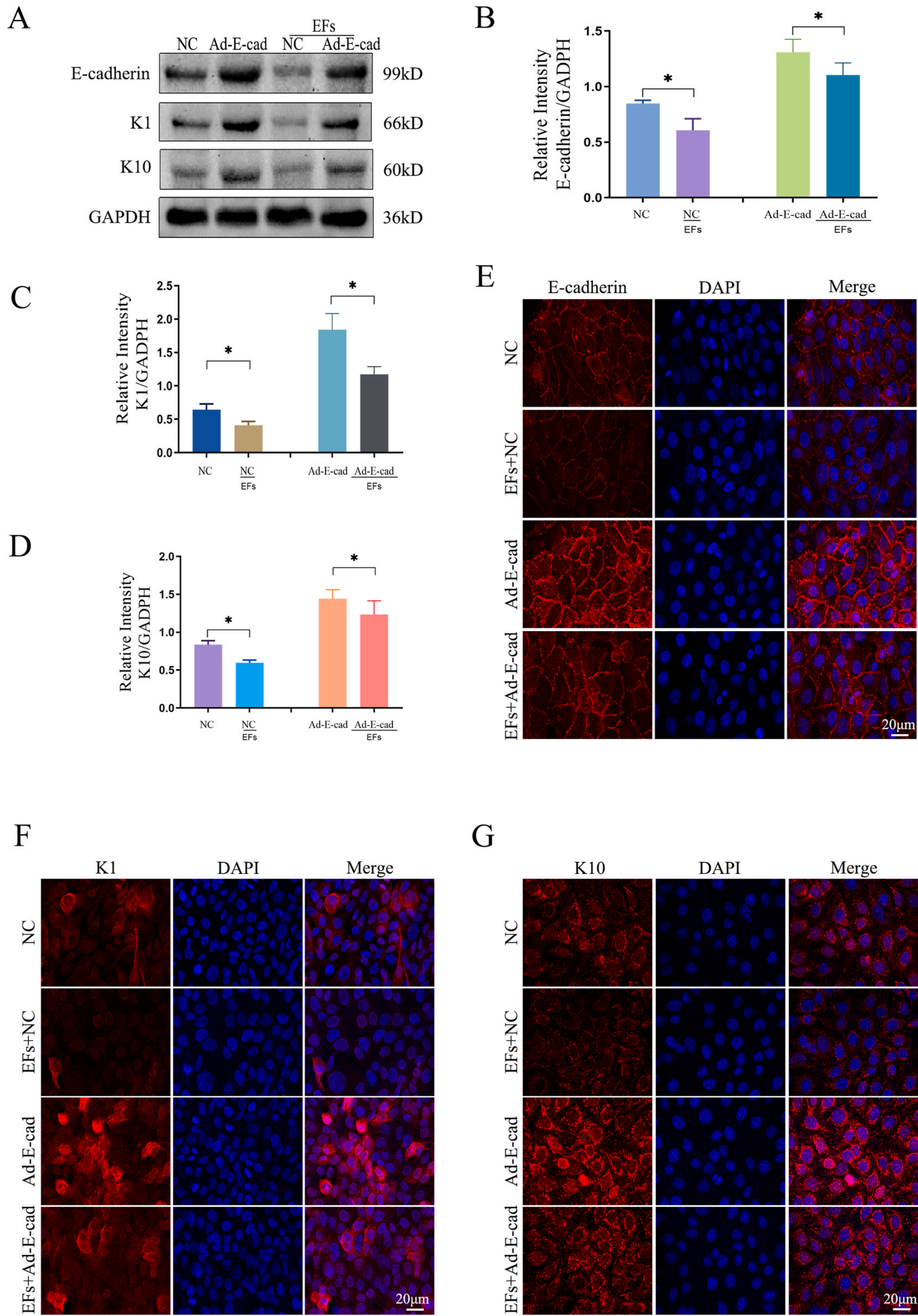


Fig. 3. E-cadherin mediated EFs-reversed keratinocyte monolayer differentiation. (A) The protein levels of E-cadherin, K1 and K10 in HaCaT cell monolayer when overexpressing E-cadherin (Ad-E-cadherin) or not and applying EFs or not were tested by Western blot. (B, C, D) And the results were quantified by relative intensity. The data was shown as the mean ± SEM (n = 3). *, $p < 0.05$ compared with negative control (NC) + EFs group. (E, F, G) Similarly, the protein and distribution of E-cadherin, K1 and K10 in cells overexpressing E-cadherin (Ad-E-cadherin) or not and applying EFs or not were observed by immunofluorescence. Bar = 20 μm.

### 2.10. Statistical analysis

The data are presented as mean ± standard error of the mean (SEM), and statistical analysis was conducted using one-way ANOVA. A p-value less than 0.05 was considered significant when performing statistical analysis.Graphs and charts were generated using GraphPad Prism and Origin software.

## 3. Results

### 3.1. The differentiation state of keratinocyte monolayer is reversed by EFs

To investigate the effect of EFs on keratinocyte differentiation, we established the HaCaT cell monolayer culture model and assessed changes in the protein levels of K1 and K10, early differentiation markers of keratinocytes, via Western blot analysis. Compared to the No EF control, exposure to EFs (200 mV/mm) led to a significant time-dependent decrease in the protein levels of K1 and K10 (Fig. 1A). Specifically, after 6 h of EFs exposure, the levels of K1 and K10 decreased by 75 % and 63 %, respectively (Fig. 1B). Immunofluorescence staining further confirmed the reduced protein levels of K1 and K10 under EFs (Fig. 1C and D). These findings indicate that EFs reverse the differentiation status of keratinocytes in cultured monolayers.

### 3.2. EFs downregulates E-cadherin in keratinocyte monolayer

E-cadherin is known to play an essential role in both migration and differentiation of keratinocytes through regulating the intercellular adhesion. To investigate the impact of EFs on E-cadherin, HaCaT monolayers were subjected to an EF with strength of 200 mV/mm. Western blot analysis showed a time-dependent reduction in E-cadherin levels, with decrease of 50 % and 71 % after exposure to EFs for 3 and 6 h, respectively (Fig. 2A and B). Furthermore, the immunofluorescence staining substantiated that the diminished of E-cadherin was particularly conspicuous on the cellular surface (Fig. 2C). These results provided substantial evidence that EFs could modulate E-cadherin in keratinocytes.

E-cadherin is known to play an essential critical role in both the migration and differentiation of keratinocytes by regulating intercellular adhesion. To evaluate the impact of EFs on E-cadherin, HaCaT monolayers were exposed to an EF with a strength of 200 mV/mm. Western blot analysis revealed a time-dependent reduction in E-cadherin protein levels, with decreases of 50 % and 71 % observed after exposure to EFs for 3 and 6 h, respectively (Fig. 2A and B). Additionally, immunofluorescence staining substantiated

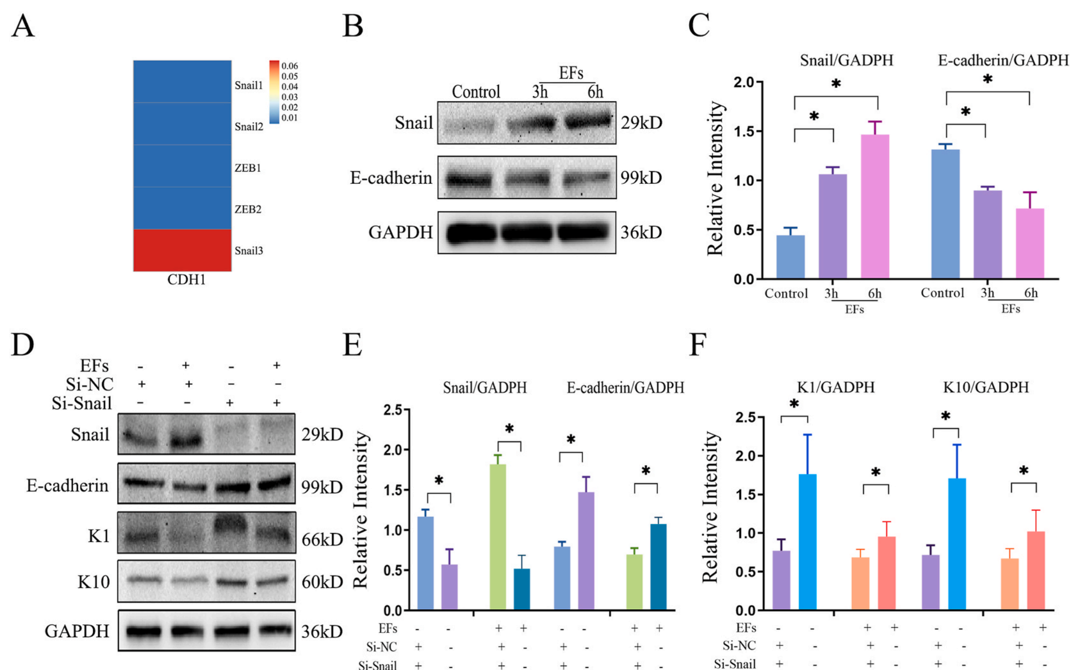


**Fig. 4.** EFs downregulated E-cadherin protein level by activating Snail. (A) The heat map of RNA-seq correlation analysis targeting Snail1, Snail2, Snail3, ZEB1, ZEB1 in HaCaT cell monolayer when electrified for 6h. (B, C) The protein levels of Snail and E-cadherin in HaCaT cell monolayer treated with no EFs (control), 3h EFs and 6h EFs were tested by Western blot and the results were quantified by relative intensity. *, $p < 0.05$ compared with no EFs (control). (D, E, F) The protein levels of Snail, E-cadherin, K1 and K10 in HaCaT cell monolayer when silencing Snail (Si-Snail) or not (Si-NC) and applying EFs or not were tested by Western blot and the results were quantified by relative intensity. The data was shown as the mean ± SEM (n = 3). *, $p < 0.05$ compared with EFs + Si-NC group.

that the decrease in protein level of E-cadherin was particularly prominent on the cellular surface (Fig. 2C). These findings provide substantial evidence that EFs can modulate E-cadherin in keratinocytes.

### 3.3. EFs reverse the differentiation of keratinocyte monolayer through modulating E-cadherin

To investigate whether E-cadherin contributes to EFs-mediated reversal of keratinocyte monolayer differentiation, recombinant adenovirus vectors overexpressing E-cadherin (Ad-E-cadherin) or silencing E-cadherin (SiRNA-E-cadherin) were constructed and used to infect keratinocytes (Fig. S1 in Supplementary material). Under normal culture conditions, protein levels of K1 and K10 were significantly higher in E-cadherin-overexpressing keratinocyte monolayers, but lower in E-cadherin-silenced keratinocyte monolayers compared to controls (Fig. S1 in Supplementary material). These results validate that E-cadherin-mediated cell-cell contacts play a role in regulating keratinocyte differentiation, aligning with previous findings [17]. We then examined the differentiation status in E-cadherin-overexpressing keratinocyte monolayers after EFs stimulation. As depicted in Fig. 3A–D, the increased protein levels of K1 and K10 in E-cadherin-overexpressing cultures were partially reversed by EFs application, accompanied by a significant suppression of E-cadherin. These findings were further supported by immunofluorescence staining (Fig. 3E–G). Thus, these results suggest that EFs reverse the differentiation of keratinocyte monolayers mainly through down-regulating E-cadherin.

### 3.4. PI3K/AKT/Snail pathway is responsible for E-cadherin downregulation in keratinocyte monolayer under EFs

To elucidate the transcriptional mechanisms underlying EFs-mediated E-cadherin down-regulation, we conducted two replicate RNA-seq experiments in the presence or absence of EFs. Correlation analysis identified the Snail family with significant correlations of 0.01 (Fig. 4A). Western blot analysis demonstrated that Snail level increased while the protein level of E-cadherin decreased under EFs treatment (Fig. 4B and C). To confirm the crucial transcriptional role of Snail in EFs-mediated E-cadherin down-regulation, cells were treated with small interfering RNA to knock down Snail. Snail knockdown significantly reversed EFs-mediated E-cadherin down-regulation in keratinocyte monolayers (0.5-fold of the Si-NC + EFs control), accompanied by increases in K1 (0.4-fold of the Si-NC

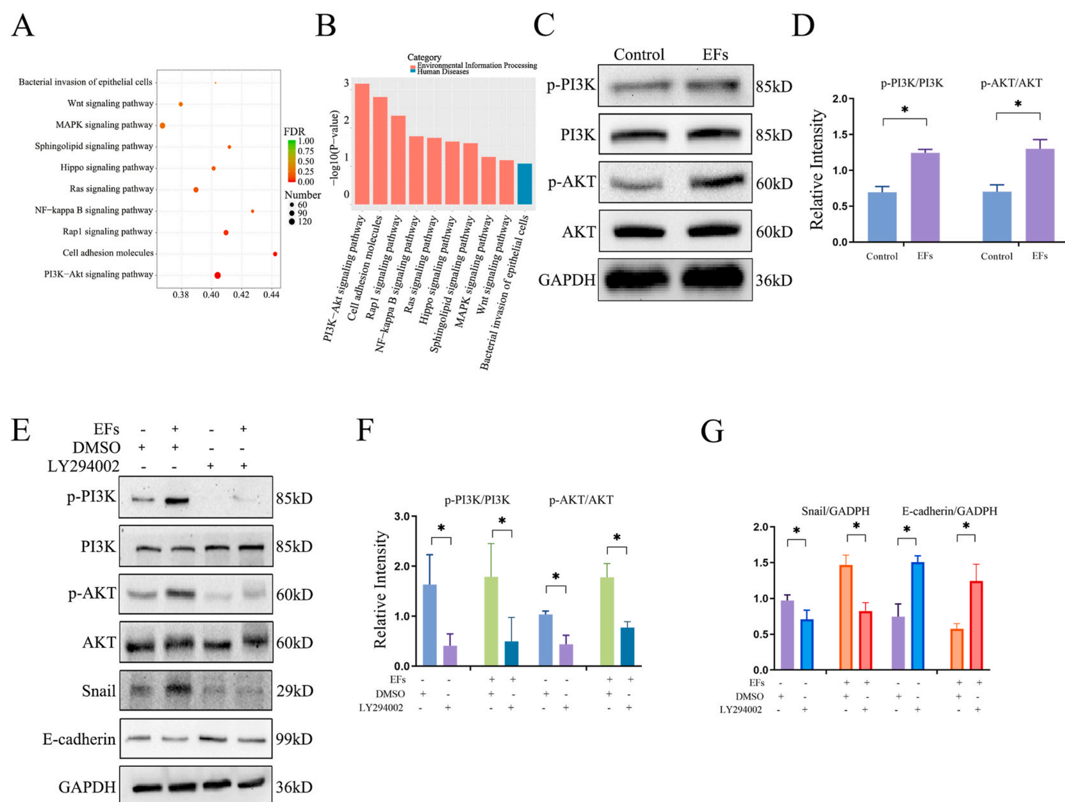


**Fig. 5.** EFs downregulated E-cadherin via the PI3K/Akt/Snail Pathway. (A) KEGG enrichment analysis of differentially expressed genes in microglia cells between the control and EFs groups. (B) KEGG pathway analysis of in HaCaT cells when electrified for 6h. (C, D) The protein levels of PI3k, p-PI3K, Akt and *p*-AKT in HaCaT cells treated with EFs or not were tested by Western blot and the results were quantified by relative intensity. *, $p < 0.05$ compared with no EFs (control). (E, F, G) The protein levels of PI3k, p-PI3K, Akt, *p*-AKT, E-cadherin and Snail in HaCaT cell monolayer when treated with PI3k inhibitor LY294002 or not (DMSO as negative control) and applying EFs or not were tested by Western blot and the results were quantified by relative intensity. The data was shown as the mean ± SEM (n = 3). *, $p < 0.05$ compared with EFs + DMSO group.

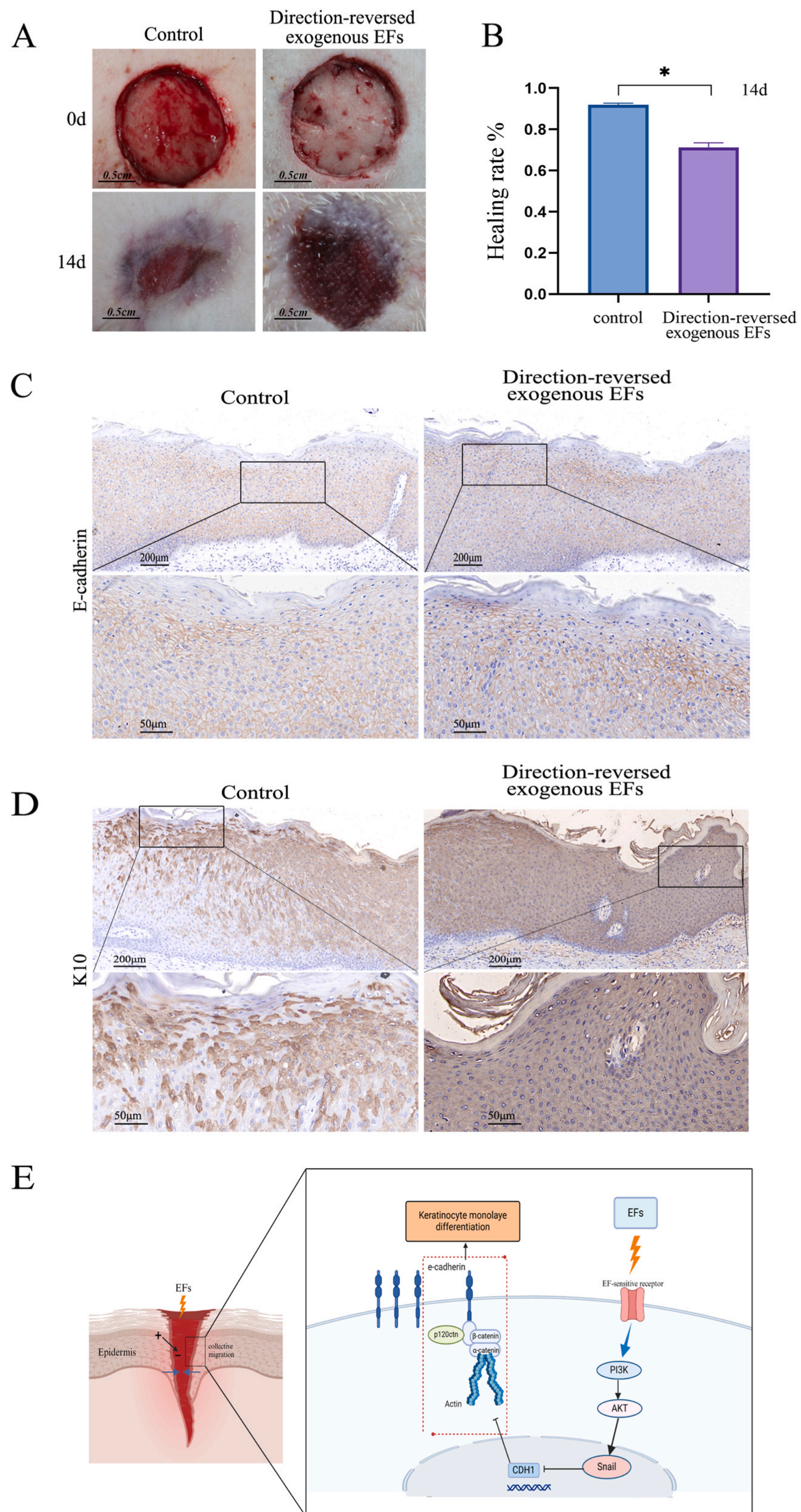


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**Fig. 6.** Direction-reversed exogenous EFs delayed wound re-epithelialization and enhanced keratinocyte monolayer differentiation. (A, B) Comparison of wound areas between controls and direction-reversed exogenous EFs groups and healing rate on 14d, bar = 0.5 cm in the bottom column. (C) An animal model of full-layer wound of balm pigs was established. A wound surface was randomly selected to apply the direction-reversed exogenous EFs or no EFs. Histology of E-cadherin in Wound tissue on 14d when applying direction-reversed exogenous EFs or not. In the upper column, bar = 200 μm and bar = 50 μm in the bottom column. (D) Histology of K10 in Wound tissue on 14d in control and direction-reversed exogenous EFs. And in the upper column, bar = 200 μm and bar = 50 μm in the bottom column. (E) Schematic diagram of EFs reversed keratinocyte monolayer differentiation by downregulating the protein level of E-cadherin via activating PI3K/AKT/Snail pathway.

\+ EFs group) and K10 (0.5-fold of the Si-NC + EFs group) respectively (Fig. 4D–F).

To investigate the upstream signal responsible for Snail induction under EFs, we conducted transcriptome sequencing to detect the enrichment of related pathways. KEGG analysis revealed that the PI3K/AKT pathway was enriched in more than 120 genes with high significance (Fig. 5A and B). Western blot analysis demonstrated that the levels of *p*-AKT and p-PI3K in keratinocyte monolayers increased after EFs exposure, indicating activation of the PI3K/AKT pathway (Fig. 5C and D). To determine if EFs down-regulate E-cadherin through the PI3K/AKT/Snail pathway, the PI3K-specific inhibitor LY294002 was used. LY294002 significantly reversed EFs-induced Snail augmentation and E-cadherin reduction in keratinocyte monolayers (Fig. 5E–G). These results suggest that activation of the PI3K/AKT/Snail pathway is crucial in the EFs-mediated reversal of keratinocyte monolayer differentiation through down-regulating E-cadherin.

### *3.5. Weakening of wound EFs lead to enhanced E-cadherin as well as earlier differentiation in newly formed epidermis in vivo*

Wound endogenous EFs are recognized as important factors that accelerate wound re-epithelialization by promoting the migration of keratinocytes [22]. Building upon our aforementioned findings, we hypothesize that wound EFs may facilitate epidermal migration by regulating its differentiation status, as low differentiation is typically associated with high motility in cells. To investigate this hypothesis, we established a full-thickness skin defect wound model in balmy pigs, and wound EFs were counteracted by applying an exogenous EF (100 mV/mm) with its direction opposite to the endogenous EFs (Fig. S3). As anticipated, weakening of wound EFs significantly delayed wound healing (Fig. 6A and B). Immunostaining results revealed that both E-cadherin and K10 in the newly formed epidermis at day 14 post-wounding were markedly higher in the reversed exogenous EFs group compared to the control (Fig. 6C and D). These findings provide in vivo evidence supporting an essential role for EFs in lowering the differentiation status of keratinocytes, thereby facilitating cell sheet migration.

## 4. Discussion

The collective migration of keratinocytes from the wound edge to the wound center is a critical step in wound healing [23]. Before this migration occurs, keratinocytes at the wound edge undergo marked changes to activate a migratory phenotype. Previous studies have suggested that wound EFs can override other microenvironmental cues and guide the directional migration of keratinocytes, yet the underlying mechanisms remain largely unclear. The findings of our current study clearly show that electric fields (EFs) reverse the differentiation status of keratinocytes by down-regulating E-cadherin through the PI3K/AKT/Snail pathway, suggesting a novel mechanism by which EFs may facilitate the migration of keratinocytes by directly controlling cell differentiation.

Keratinocytes in the healthy skin epidermis typically exhibit a layered structure, with K5 and K14 predominantly expressed in basal keratinocytes, while K1 and K10 are characteristic of differentiated keratinocytes in the stratum spinosum [24]. During wound healing, keratinocytes at the wound edge initiate collective migration with notable changes conducive to a migratory state, including loosening of intercellular adhesions and reversal of differentiation status. Upon completion of healing, the new epidermis ceases migration and resumes a program of stratification and differentiation essential for functional epidermal integrity, resembling normal skin [21,25]. Endogenous wound EFs, which emerge immediately after injury, are recognized as key factors directing the collective migration of keratinocytes towards the wound center [3,4]. These wound EFs gradually diminish throughout the healing process and eventually dissipate upon completion of healing [26]. Remarkably, the changes in wound EFs during healing exhibit a highly spatial and temporal overlap with the phenotype changes of keratinocytes. However, whether EFs contribute to these phenotype changes that favor cell sheet migration during the healing process remains elusive. High motility is often correlated with low cell differentiation. As an immediate microenvironmental factor post-injury, we hypothesize that wound EFs may act as an initial stimulus to induce low differentiation of keratinocytes in a wound. Indeed, EFs have been demonstrated to regulate differentiation in various cell types, with the exact role depending on the specific scenarios and parameters of the applied EFs [27–30]. In this study, utilizing the HaCaT monolayer culture model, we observed a significant, time-dependent decrease in the early differentiation markers of keratinocytes, K1 and K10, following EFs treatment, providing evidence for EFs in reversing keratinocyte differentiation.

Cell sheets are fundamentally different from isolated cells, as mechanical couplings between cells via intercellular adhesion are essential. In epidermal keratinocytes, E-cadherin, the primary member of the classical cadherin family, holds significant importance within this framework.It has been identified that a low level of E-cadherin favors the collective migration of epithelial sheets [31], although disruption of E-cadherin-mediated adhesion leads to a loss of coordinated migration of cell sheets [16]. Moreover, E-cadherins are widely involved in the regulation of cell differentiation [13,32]. E-cadherin interactions have been shown to be necessary for Langerhans cell differentiation and the hepatic-specific differentiation of mesenchymal stem cells [33,34]. Notably, E-cadherin-mediated adhesion plays a crucial role in keratinocyte differentiation [13]. In our study, we observed that the level of plasma membrane E-cadherin positively correlates with the differentiation status of keratinocyte monolayers (Fig. S1). Interestingly, EFs

treatment resulted in a time-dependent decrease in E-cadherin in keratinocyte monolayers, accompanied by suppression of differentiation (Fig. 2). Furthermore, enhanced differentiation in E-cadherin-overexpressed monolayers could also be reversed by EFs application (Fig. 3). These results suggest that E-cadherin functions as a pivotal effector in EFs-reversed differentiation of keratinocyte monolayers. This conclusion was supported by our in vivo study, where disruption of wound endogenous EFs by reversed exogenous EFs promoted newly formed epidermal differentiation with upregulation of E-cadherin (Fig. 6).

Snail, a zinc finger-containing transcription factor, was initially discovered in Drosophila as a suppressor of shotgun (an E-cadherin homologue) [35]. Its pivotal role in regulating adhesion junction proteins, particularly E-cadherin, in tumor invasion and epithelial-mesenchymal transition has been firmly established [36,37]. Snail has been shown to promote epithelial-to-mesenchymal transitions during tumor development and wound healing [38]. Transfection of Snail cDNA into MDCK cells resulted in increased cell motility and decreased transcription of E-cadherin [36]. However, the role of Snail in keratinocyte differentiation remains poorly understood. In this study, we confirmed that EFs downregulate E-cadherin via Snail through RNA-seq correlation analysis and Western blotting. Snail knockdown abolished EFs-induced E-cadherin reduction and differentiation suppression in keratinocyte monolayers, revealing a Snail-dependent transcriptional regulatory mechanism involved in the modulation of keratinocyte differentiation under EFs (Fig. 4D–F).

Previous studies have indicated that Snail could be regulated by ERK signaling [39]. However, our findings suggest that although EFs activate ERK, it is not the primary candidate for Snail induction (Fig. S2). Conversely, we identified a significant relationship between Snail augmentation and the activation of PI3K/AKT signaling under EFs. The PI3K/AKT signaling pathway governs multiple biological processes, including the cell cycle, apoptosis, angiogenesis, motility, and cell-cell adhesion [40]. Numerous studies on cancer have reported that Snail is a downstream target of PI3K/AKT signaling and facilitates the metastasis of malignant tumors [41,42]. In our study, inhibition of PI3K abolished the activation of AKT, the upregulation of Snail, and the downregulation of E-cadherin induced by EFs (Fig. 5E–G). This suggests that EFs downregulate E-cadherin through the PI3K/AKT/Snail pathway, thereby reversing the differentiation status of keratinocyte monolayers. It is noteworthy that PI3K/AKT also plays a direct role in EFs-induced cell migration during wound healing. Exposure of both keratinocytes and neutrophils to EFs activates the PI3K/AKT signaling pathway, which triggers asymmetric intracellular signal cascades and promotes directed migration consequently [6]. Therefore, PI3K/AKT may function as a common signaling pathway orchestrating the diverse cellular behaviors induced by EFs.

We have revealed an unexpected role of EFs in the regulation of keratinocyte differentiation. While EFs-induced collective migration of keratinocytes has been well established previously, we provide evidence showing that EFs reverse keratinocyte differentiation by downregulating E-cadherin through the activation of the PI3K/AKT/Snail pathway. This low differentiation state is beneficial for cell migration. Therefore, our study proposes a novel working model for wound EFs in the process of wound healing, whereby they promote epidermal migration by inducing a state of reduced cellular differentiation (Fig. 6E).

## 5. Conclusion

In conclusion, our data, combined with existing research, supports a pattern wherein EFs activate the directional migration of keratinocyte monolayers by reversing keratinocyte differentiation. Furthermore, our findings highlight the significant role of EFs in reversing keratinocyte differentiation by downregulating E-cadherin through the activation of the PI3K/AKT/Snail pathway.

## Data availability statemen

All data generated during this study are included in this article and supplementary materials, Raw data is available upon reasonable request.

## CRediT authorship contribution statement

**Chao Wu:** Writing – original draft, Methodology, Formal analysis. **Xu Chen:** Writing – review & editing, Software, Methodology, Formal analysis, Data curation. **Wanqi Huang:** Writing – review & editing, Software, Methodology, Data curation. **Jinrui Yang:** Project administration, Investigation. **Ze Zhang:** Software, Data curation. **Jie Liu:** Funding acquisition, Formal analysis. **Luojia Liu:** Methodology, Investigation. **Ying Chen:** Methodology, Formal analysis, Data curation. **Xupin Jiang:** Writing – review & editing, Supervision, Resources, Funding acquisition, Formal analysis, Conceptualization. **Jiaping Zhang:** Supervision, Funding acquisition, Formal analysis.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33069.

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